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THE KINETICS OF TRYPSIN DIGESTION.

I. Experimental Evidence Concerning the Existence of an Intermediate Compound.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 20, 1923.)

The anomalies of enzyme action have frequently been accounted for by the assumption of an intermediate compound between the enzyme and the substrate. It was shown, however, by Nelson and Hitchcock[1] that in the case of invertase the experiments could not be accurately calculated on this basis. The writer found with trypsin[2] that although the effect of varying the substrate concentration could be accounted for, the effect of varying both the substrate and inhibiting substances could not, provided the law of mass action was assumed to hold. The question is one of such importance from the view of all enzyme reactions, however, that it seems important to obtain direct experimental evidence in regard to the existence of such a compound.

The statement is frequently made that the substrate protects the enzyme from heat inactivation and this has been considered as evidence for the existence of a compound between the substrate and enzyme. It has been found,[3] however, that this protective action does not become manifest at once but only after the enzyme and substrate have been in contact, when it becomes more and more pronounced. It can further be shown that the products of digestion afford very marked protection and that this effect is apparent at once. It appears probable, therefore, that the protective action of the substrate solution is not due to the substrate itself but to the products formed by the action of the enzyme and that this is the reason for the delay in the protective action.

[1] Nelson, J. M., and Hitchcock, D. I., *J. Am. Chem. Soc.*, 1921, xliii, 2632.
[2] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 487.
[3] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv. 261.

A second experimental test is furnished by the action of the enzyme on two substrates at once. If the enzyme is combined with one, it evidently cannot act on the other. This experiment has been performed by the writer[2] on a mixture of casein and gelatin with the result that the rate of reaction of the mixture was the sum of the two independent reactions. In this experiment the sum of the actions was measured which renders the result more complicated. By changing the method, however, it is possible to make a direct experimental comparison and this has been done in the present paper. This is made possible by the fact that casein is insoluble in trichloroacetic acid[4] and may therefore be determined in the presence of gelatin, which is soluble. The experiment, therefore, consists in adding the same amount of trypsin to two solutions each containing 2.5 per cent casein and one of which contains in addition 3 per cent of gelatin. In order to avoid complicating effects due to the retarding action of the products of the reaction, it is necessary to use a large amount of trypsin.

Experiment I. The Effect of Gelatin on the Rate of Hydrolysis of Casein. Casein Solution.—2.5 gm. of casein dissolved in M/10 phosphate buffer pH 7.6, the pH readjusted to 7.6, and the solution made up to 100 cc.

Casein-Gelatin Solution.—2.5 gm. of casein and 2.5 gm. of gelatin dissolved and made up to 100 cc. as above. pH 7.6.

Both solutions brought to 37°C. and 10 cc. of trypsin added to each. 2 cc. samples pipetted into 10 cc. of 5 per cent trichloroacetic acid at time intervals shown in the figure. These solutions were allowed to stand 1 hour, centrifuged, the residue redissolved in alkali, and reprecipitated with trichloroacetic acid. The precipitate was then dissolved in alkali, made up to 10 cc., and 1 cc. analyzed for total nitrogen by the micro Kjeldahl method of Folin and Farmer.[5]

Experiments[2] in which the concentration of gelatin alone varied show that the amount digested by a given amount of trypsin is practically independent of the gelatin concentration if the latter is greater than 2 per cent. On the intermediate compound theory this would be accounted for by supposing that the enzyme is saturated with substrate. It is evident that if the trypsin is saturated with gelatin, it cannot act on the casein. The experiment showed, however, that the rate of casein digestion is unaffected by the presence of the gelatin.

[4] Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

[5] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

This experiment shows conclusively that the anomalous results obtained in varying the gelatin concentration cannot be due to the

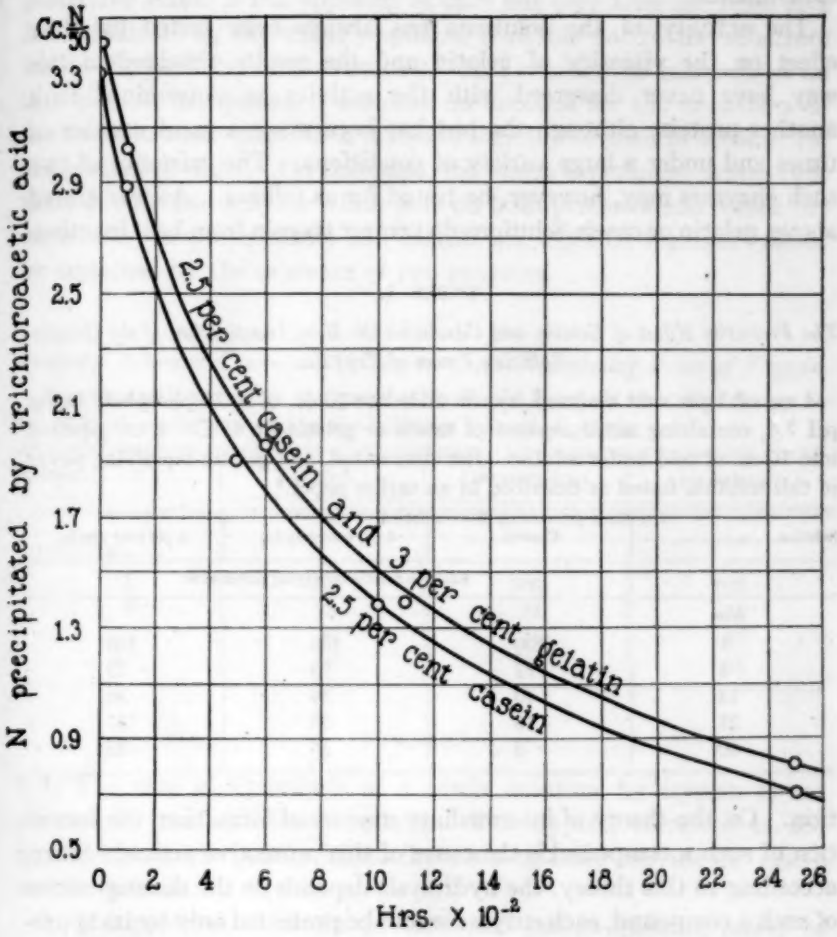

FIG. 1. The rate of digestion of casein alone and in the presence of 3 per cent gelatin. Nitrogen precipitated by trichloroacetic acid (ordinates) is plotted against the time of reaction (abscissæ).

formation of a compound between the enzyme and gelatin, unless it be further assumed that there are present two enzymes, one of which attacks the gelatin and one the casein. The writer has never suc-

ceeded in obtaining the slightest evidence for the existence of more than one proteolytic enzyme in the trypsin (Fairchild's) used in these experiments.

The activity of the solutions has always been tested by their effect on the viscosity of gelatin and the results obtained in this way have never disagreed with the activity as determined with another protein, although the test has been made a great number of times and under a large variety of conditions. The existence of two such enzymes may, however, be tested for as follows. As was stated above, gelatin or casein solutions do protect trypsin from heat inactiva-

TABLE I.

The Protective Effect of Gelatin and Casein on the Heat Inactivation of the Gelatin-Splitting Power of Trypsin.

1 cc. of 2 per cent dialyzed trypsin added to 10 cc. of M/10 phosphate buffer pH 7.6, containing noted amount of casein or gelatin at 45°C. 1 cc. pipetted into 10 cc. of cold buffer solution after time noted, and gelatin-liquefying power of this solution tested as described in an earlier paper.[6]

Solution.....	Control.	4 per cent gelatin.	4 per cent casein.
	Relative gelatin-liquefying power after		
min.			
1	100	100	100
5	72	73	73
13	40	54	56
25	18	38	37
60	6	35	33

tion. On the theory of intermediate compound formation, the formation of such a compound is the cause of this protective action. Since, according to this theory, the hydrolysis depends on the decomposition of such a compound, each enzyme would be protected only by its appropriate substrate. Casein, therefore, should not protect the gelatin-splitting enzyme, and gelatin should not protect the casein-splitting enzyme. The experiment shows, however, that this is not the case. The gelatin-splitting enzyme is protected by casein, and the casein-splitting enzyme is protected by gelatin. The result of such an

[6] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

experiment is shown in Tables I and II. The experiments show the peculiarity mentioned at the beginning of this paper; namely, that the protective action is not apparent at once but only after the lapse of a few minutes. In the writer's opinion, it is due not to the substrate but to the products of the reaction. If, however, the existence of an intermediate compound is assumed to account for the anomalies of the concentration effect, then it should logically be used to account for the protective effect. On this basis, the above experiment proves that there is only one enzyme which acts on both products and hence the experiment in which casein and gelatin are digested together cannot be explained by the existence of two enzymes.

TABLE II.

Protective Action of Casein and Gelatin on Casein-Hydrolyzing Power of Trypsin.

Experimental procedure the same as in Table I, except that the change in viscosity of casein was used as the indicator instead of gelatin.

Solution.....	Control.	4 per cent gelatin.	4 per cent casein.
	Relative casein-hydrolyzing power after		
min.			
1	100	100	100
5	73	74	73
25	20	44	47
65	7	35	33

SUMMARY.

1. The rate of hydrolysis of a casein solution by trypsin is not affected by the addition of gelatin. The trypsin, therefore, is not combined with the gelatin unless there is a separate enzyme for casein and for gelatin.

2. The presence of casein protects the gelatin-splitting power of trypsin from heat inactivation, and the presence of gelatin protects the casein-splitting power from heat inactivation.

3. It does not seem possible to account for both the above results by the assumption of an intermediate compound between enzyme and substrate, since, in order to account for the first result, a different enzyme must be assumed for each protein, while, to account for the second result, it must be assumed that the same enzyme attacks both.

THE KINETICS OF SENESCENCE.

By SAMUEL BRODY.

(From the Department of Dairy Husbandry, University of Missouri, Columbia.)

(Received for publication, October 8, 1923.)

The question of why we grow old and die is gradually being answered with the accumulation of data.[1]

While this question is a very old one, the wide scientific interest in this subject dates from the time of Weismann's discussion on life and death.[2] Weismann popularized the fact first pointed out by Bütschli that unicellular organisms may be considered as immortal since they will live and multiply indefinitely in appropriate media which are frequently renewed.

The observation by Leo Loeb[3] that cancer cells, which are considered as merely modified somatic cells, can be grown indefinitely when transplanted successively from the old host to younger animals was the next contribution to this subject. This led to the conclusion that cancer cells must be considered immortal and he generalized this for all forms of somatic cells of multicellular animals. The proof of this was furnished by the experiments on tissue culture, as gradually developed by Leo Loeb,[4] Harrison,[5] Burrows,[5] and Carrel

[1] The literature on senescence was recently fully summarized in the following two monographs. Pearl, R., The biology of death, Monographs on experimental biology, New York and London, 1922. Robertson, T. B., The chemical basis of growth and senescence, Monographs on experimental biology, Philadelphia and London, 1923.

[2] Weismann, A., Ueber die Dauer des Lebens, Jena, 1882; Ueber Leben und Tod, Jena, 1884.

[3] Loeb, L., *J. Med. Research*, 1901, vi, 28.

[4] Loeb, L., Über die Entstehung von Bindegewebe, Leucocyten, und rothen Blutkörperchen aus Epithel und über eine Methode isolierte Gewebsteile zu züchten, Chicago, 1897. Quoted by Loeb, J., The organism as a whole from a physicochemical viewpoint, New York and London, 1916.

[5] Harrison, R. G., *J. Exp. Zool.*, 1910, ix, 787; *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 63. Burrows, M. T., *J. Exp. Zool.*, 1911, x, 63; *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

and Burrows, and Carrel.[6] These modifications enabled Carrel,[6] and Carrel and Ebeling[14] and other collaborators to grow several types of tissues *in vitro* some of them for many years thus substantiating the suggestion of the potential immortality of somatic cells of higher organisms.

Moore[7] who, at the suggestion of Loeb, investigated the temperature coefficient for the duration of life for the hydranth of a tubularian, found the temperature coefficient to be of the order of a chemical reaction. The observations of Gudernatsch[8] that the duration of the tadpole stage can be controlled by thyroid feeding, and of Morse[9] that the same results may be obtained by feeding iodized amino-acids, add further support to the theory that a chemical substance may control the duration of life.[10]

Jacques Loeb started in 1908[11] an investigation on the temperature coefficient of the duration of life and the cause of natural death which was continued by him in collaboration with Northrop[12] on aseptic cultures of *Drosophila*. In these organisms all accidental causes of death were avoided and it could be shown that there is a temperature coefficient for the duration of life of these organisms which is of the order of magnitude of that of a chemical reaction. The duration of life of the aseptic flies of *Drosophila* was trebled when the temperature was lowered by 10°C. The authors arrived at the conclusion that the duration of life of organisms is the time required to complete a chemical reaction or a series of chemical reactions, resulting either in the production of toxic substances in sufficient

[6] Carrel, A., and Burrows, M. T., *Compt. rend. Soc. biol.*, 1910, lxix, 293, 298, 299, 328, 365; *J. Exp. Med.*, 1911, xiii, 387; 1911, xiv, 244. Carrel, A., *J. Exp. Zool.*, 1911, x, 63; *J. Exp. Med.*, 1911, xiii, 416; 1912, xv, 393, 516; 1913, xvii, 14; 1913, xviii, 287.

[7] Moore, A. R., *Arch. Entwcklngsmechn. Organ.*, 1910, xxix, 287.

[8] Gudernatsch, J. F., *Zentr. Physiol.*, 1912, xxvi, 323; *Arch. Entwcklngsmechn. Organ.*, 1912–13, xxxv, 457; *Am. J. Anat.*, 1913–14, xv, 431.

[9] Morse, M., *J. Biol. Chem.*, 1914, xix, 421.

[10] *Cf.* also the recent interesting discussion on Amphibian metamorphosis, by Huxley, J., *J. Hered.*, 1922, xiii, 349; 1923, xiv, 3.

[11] Loeb, J., *Arch. ges. Physiol.*, 1908, cxxiv, 411.

[12] Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 382; *J. Biol. Chem.*, 1917, xxxii, 103. Loeb, J., *Scient. Month.*, 1919, ix, 578.

quantity to make us grow old and ultimately kill us, or in the destruction of some needful substance, or both. The idea of Metchnikoff[13] that these substances are produced by bacteria in the intestine does not hold for these experiments, since the flies used were free from microorganisms.

Finally the latest support of Loeb's theory of senescence is furnished by the observations of Carrel and Ebeling[14] that blood serum from a young animal is a better nutrient medium for growth of tissues *in vitro* than serum from an older animal; and that diluting the serum from the old animal, that is decreasing the concentration of the hypothetical toxic substance or substances, improves it as a medium for the growth of the tissues *in vitro*.

Assuming the above conclusion, that senescence and death are due to the accumulation of a toxic substance or substances in the body represents the fact, then the most important problem relating to senescence is to determine the properties and manner of formation of this substance or of these substances.

An indirect method often employed by physical chemists and biologists in studies on the manner of formation and of the properties of a substance or substances limiting a process is to determine the kinetics, or time relations, of the process. This method has, for example, been found useful in the study of radioactive substances;[15] in the study of injury, recovery, and death of plant tissues;[16] and in the study of growth of plants and animals.[1] It may be that a knowledge of the kinetics of senescence will likewise prove useful in getting an insight into the process of senescence, and getting an idea concerning the nature of the agencies limiting the process of senescence. Thus, if the course of senescence is found to follow the course of chemical reactions *in vitro*, it may be considered to substantiate the theory that senescence is a physicochemical process limited by physicochemical agencies.

[13] Metchnikoff, E., The prolongation of life, New York, 1908.

[14] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

[15] *Cf.* Rutherford, E., Radioactive substances and their radiations, Cambridge, 1913.

[16] Osterhout, W. J. V., Injury, recovery, and death, in their relation to conductivity and permeability, Monographs on experimental biology, Philadelphia and London, 1922.

Data on the course of senescence, and their analyses from this physicochemical standpoint have already been presented as regards the factors limiting milk production with age in the dairy cow[17] and the factors limiting egg production with age in the domestic fowl.[18] It was shown that the decline in the speed of egg production with age in the domestic fowl may be represented by the same exponential relation which is used to represent the decline in the speed of a monomolecular chemical reaction with time

$$Y = Ae^{-kt} \tag{1}$$

in which Y represents the number of eggs laid per year, which is taken as an index of vitality, or reciprocal of senescence, of the fowl at the age, t. This equation is taken to represent the course of decline of vitality with age when not complicated by growth because the domestic fowl is thought to be sexually mature during her first laying year.

The case of change of milk production with age in the dairy cow[17] is somewhat different; milk secretion begins at about 2 years of age, while the cow does not reach full body weight until 8 or 9 years. Milk production at any age, is therefore taken to represent the resultant of two processes, growth and senescence. The speed of growth of the dairy cow after the age of 2 years was found to decline exponentially with age as represented by equation (1)[19] which, as pointed out, also represents the decline in vitality with age in the domestic fowl. It therefore appears that growth and senescence both follow the same exponential law—the law of monomolecular change in chemistry; and that the two processes are simultaneous and consecutive. It was in fact found[17] that the course of milk production with age in the dairy cow can be represented by the equation of two simultaneous consecutive reactions

[17] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1923–24, vi, 31.

[18] Brody, S., Henderson, E. W., and Kempster, H. L., *J. Gen. Physiol.*, 1923–24, vi, 41.

[19] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 445.

$$Y = Ae^{-k_1 t} - Be^{-k_2 t} \qquad (2)$$

in which Y is the milk production which is taken as an index of vitality at the age, t. The first term on the right side of the equation represents the course of decline of milk secretion with age due to the process of senescence; the second term represents the declining increase in vitality with age due to the process of growth. If the above premises and reasoning are correct, then equations (1) and (2) should represent respectively the course of vitality (reciprocal of senescence) when not complicated by growth, and vitality when complicated by growth. It is the purpose of this communication to test this idea by attempting to fit equations (1) and (2) to certain published data which are thought to indicate the course of vitality or senescence with age.

The most interesting data on the course of vitality with age are those obtained by Carrel and Ebeling[14] on the rate of growth, and the duration of life of fibroblasts cultivated *in vitro*, using plasma from the domestic fowl as a culture medium. They found that there was a definite relation between the age of the animal and the amount of new tissue produced in its plasma in a given time. The duration of life of the fibroblasts decreased in a similar way with the age of the fowl from which the plasma was used. While only a few experiments have been made, which may turn out to be statistically insufficient for drawing conclusions concerning the significance of the agreement between computed values as obtained from equation (1) and observed values, nevertheless, because of the importance of these data, it seemed worth while to attempt to roughly determine the mathematical relation connecting the duration of life of the fibroblasts *in vitro* and the decline in ovulation, with the age of the fowl from which plasma was used as a culture medium. Such a comparison was made, and it is shown in Fig. 1. In Fig. 1 it is seen that the course of duration of life of fibroblasts with age and also the course of ovulation with age follow the same course as represented by equation (1).

As pointed out in the same paper by Carrel and Ebeling[14] the relation between the age of an animal and the speed of healing of its wound is probably closely allied to the effect of age of the animal

from which the plasma is used as a culture medium, on the speed of growth of the fibroblasts. Both phenomena are probably ultimately dependent on the condition of the blood. Fig. 1 also shows that the curve of healing of a wound 40 sq. cm. in area as interpolated from Text-fig. 1 of the paper by du Noüy[20] also follows the course represented by equation (1).

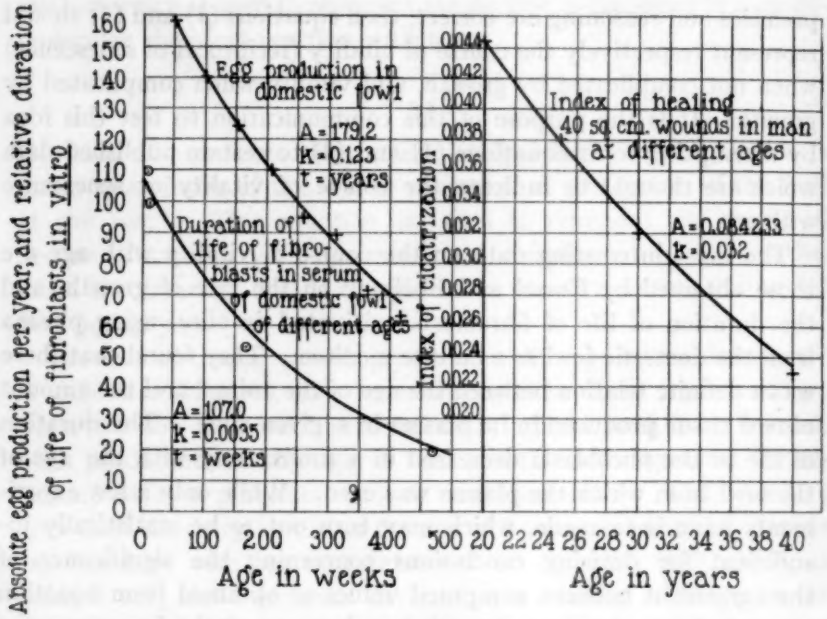
Absolute egg production per year, and relative duration of life of fibroblasts in vitro
Egg production in domestic fowl
A = 179.2
k = 0.123
t = years
Duration of life of fibroblasts in serum of domestic fowl of different ages
A = 107.0
k = 0.0035
t = weeks
Index of cicatrization
Index of healing 40 sq. cm. wounds in man at different ages
A = 0.084233
k = 0.032
Age in weeks
Age in years

FIG. 1. The effect of age on the vitality of the domestic fowl as measured by the duration of life of fibroblasts in its serum and by its egg production; also the vitality of man as measured by the rate of healing of 40 sq. cm. wounds at different ages. The crosses and circles are the observed values; the curves represent the equation

$$y = Ae^{-kt}$$

The values A and k are indicated near the curves, the source of data is given in the text.

In addition to these data obtained under conditions which are more or less under control there are available statistical data which

[20] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 463.

seem suitable for measuring the course of senescence. The mortality data on man are of course the largest body of this type of data. It seems reasonable to assume, that other conditions being the same, the greater the degree of senescence, the greater would be the chances of breaking down of some vital organ-system due to a given unfavorable exposure. The course of mortality under such conditions should therefore form an index of the course of senescence. All other conditions are not, however, the same throughout life. Many types of mortality such as those due to occupational accidents and diseases; to the maternal state; to infectious diseases, as tuberculosis, which act selectively during the earlier years for reasons other than senescence, are of course independent of senescence as such. On the other hand there are types of mortality due to the breaking down of organ-systems subjected to approximately uniform environmental conditions throughout life and which seem to be closely associated with the degree of senescence. As examples of these latter types of mortality may be mentioned mortality due to cerebral hemorrhage and apoplexy; to diseases of the arteries; to Bright's disease; to cancer; and to certain infectious diseases to which the exposure is approximately the same throughout life, which do not develop an immunity, and which do not act cumulatively; for example, pneumonia. Of course the important factor of heredity must be left out in a study of statistical data on man. In spite of leaving out the factor of heredity and of other obvious defects of the data, the course of mortality due to these diseases follows rather closely an exponential course with age as shown in Fig. 2. The straightness of the mortality curves on this semilogarithmic paper indicates that the speed of mortality changes in geometrical progression with age which is the fact represented by equation (1).

The curves in Fig. 2 represent the specific mortality data only after about 15 years of age. Up to this age, the specific mortality decreases, which decrease is thought to be principally due to increasing vitality; for experience shows that the same child can withstand less favorable conditions at the age of 10 to 15 years than it could when it was younger, for example, between birth and 1 year of age. The whole life curve of vitality as measured by the reciprocal of mor-

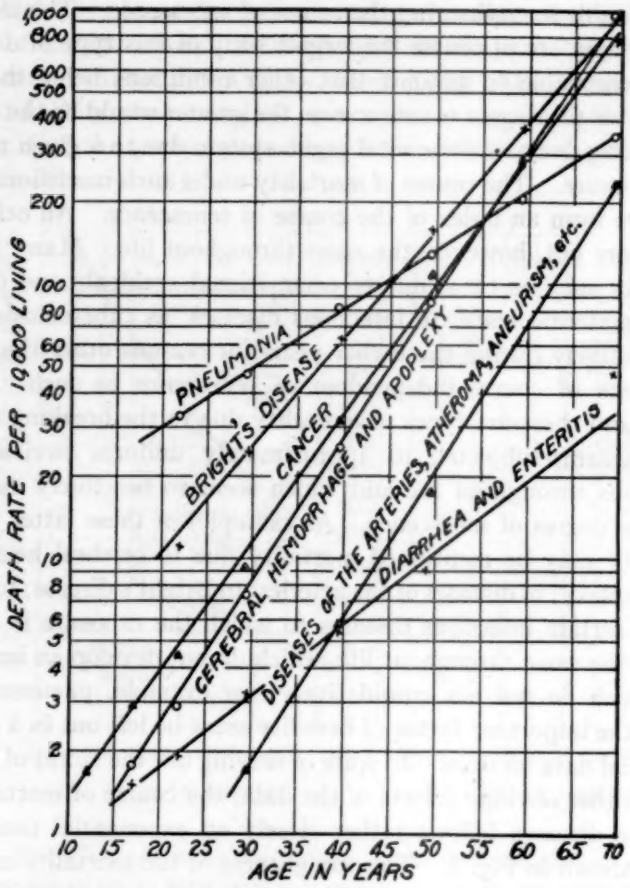

FIG. 2. The effect of age on the degree of senescence in man as measured by the specific death rates between 15 and 70 years. The data represent deaths per 10,000 persons exposed at the given ages which are based on the general male population of the United States Registration Area 1910–15 as given by Dublin and coworkers.[21] The straightness of the curves on this semilogarithmic paper shows that the course of mortality with age follows an exponential law. (From the curves, the mortalities due to cancer, to cerebral hemorrhage and apoplexy, and to diseases of the arteries, atheroma, aneurism, etc., are doubled about every 6 years; mortality due to Bright's disease is doubled about every 8 years; mortality due to enteritis and pneumonia are doubled, respectively, about every 12 and 14 years.)

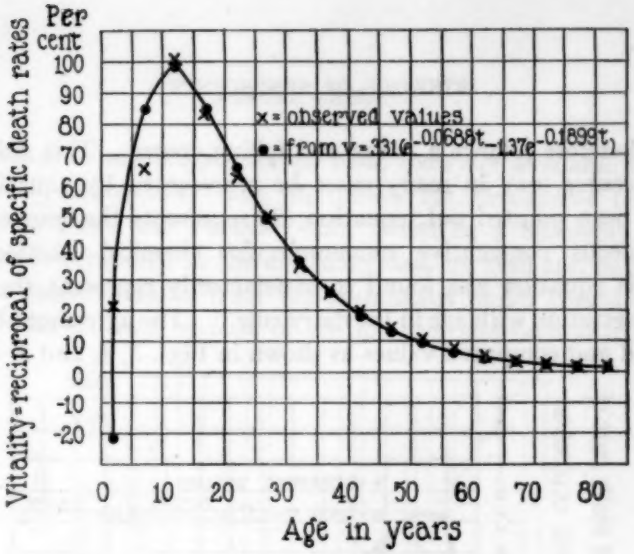

FIG. 3. The rising and declining course of vitality with age as measured by the reciprocals of specific death rates due to the breaking down of the nervous system and sense organs expressed in terms of percentage of maximum vitality. The values for vitality which are based on the mortality in the United States Registration Area exclusive of North Carolina in 1910 were computed from Group VII of Pearl's classification. The original data are given in Table IX in Pearl's monograph,[1] × = observed values; ● = computed from $v = 331\ (e^{-0.0688t} - 1.37e^{-0.1899t})$.

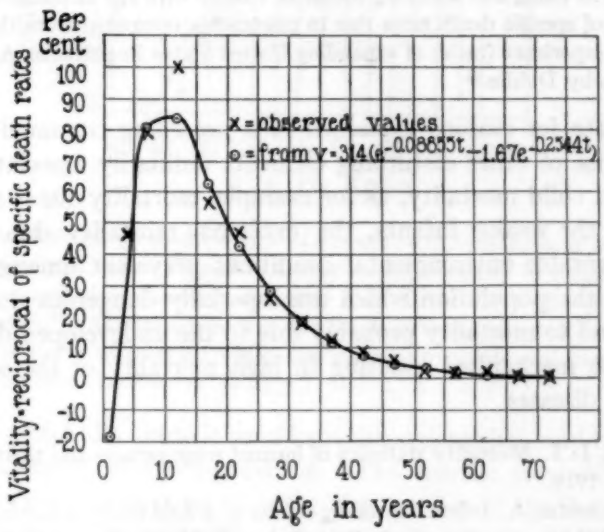

FIG. 4. The rising and declining course of vitality with age as measured by the reciprocals of specific death rates due to the breaking down of the kidney and related excretory organs (Group IV of Pearl's classification). For source of data see legend, Fig. 3. × = observed values; ⊙ = $v = 314\ (e^{-0.08855t} - 1.67e^{-0.2544t})$.

tality therefore follows a rising and falling course. This rising and falling course may in many cases be represented by equation (2). As has been pointed out, equation (2) represents the course of two simultaneous consecutive monomolecular chemical reactions, and that this equation was found to satisfactorily represent the course of milk secretion with age in the dairy cow.[17] The agreement between observed and computed values as shown in Figs. 3, 4, and 5 is only

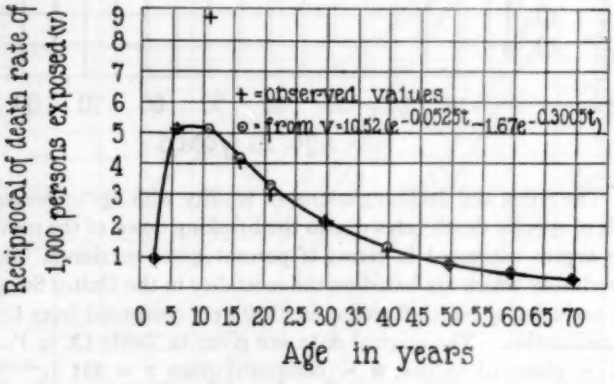

FIG. 5. The rising and declining course of vitality with age as measured by the reciprocals of specific death rates due to pneumonia computed from the general population experience (male) of expanding United States Registration Area 1910–15 as given by Dublin.[21]

approximate for reasons explained in a preceding communication,[17] and because of other disturbing elements ordinarily associated with infant and child mortality, as for example, mortality due to selective action on the weaker infants, the excessive mortality due to certain unfavorable environmental conditions prevalent among certain classes of the population which are especially dangerous during infancy;[22] and to mortality probably due to the undeveloped defensive or immune mechanism resulting in high mortality of the so called children's diseases.

[21] Dublin, L. I., Mortality statistics of insured wage earners and their families, New York, 1919.

[22] *Cf.* Rochester, A., Infant mortality results of a field study in Baltimore, Md. Based on births in one year, *U. S. Dept. Labor, Children's Bureau, Pub. No. 119*, 1923. *Cf.* also Holmes, S. J., and Goff, J. C., Eugenics in race and state, *2nd Internat. Cong. Eugenics, 1921*, 1923, ii, 233.

In addition to mortality data on man, data are available on the mortality of *Drosophila*. The life tables for *Drosophila* recently published by Pearl and Parker[23] while based on a relatively small population as compared to the mortality data on man are, nevertheless, even more valuable from the standpoint under consideration than the data on man because of the uniform and favorable condi-

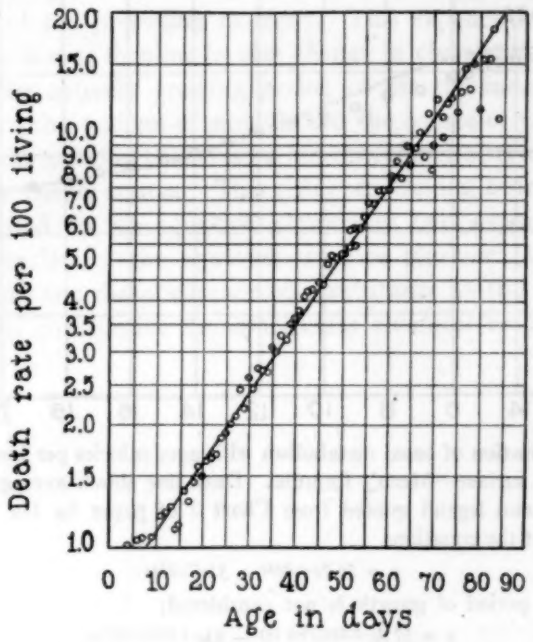

FIG. 6. The effect of age on the degree of senescence in *Drosophila* as measured by the specific death rates. The data represent the mortality of females computed (by the method explained in the text) from Table III of the paper by Pearl and Parker.[23] The straightness of the curve on this semilogarithmic paper shows that the curve of mortality with age follows an exponential law. (The mortality is doubled about every 18 days.)

tions under which the animals were kept throughout life, and because of the homogeneity of the population. The specific mortalities for *Drosophila* were obtained by dividing the number of deaths during each day as given in Table III of the paper by Pearl and Parker[23]

[23] Pearl, R., and Parker, S. L., *Am. Naturalist*, 1921, lv, 481.

by the number living at the beginning of the day interval. The results shown in Fig. 6 are seen to follow an exponential course as represented by equation (1). The deviations of the observed values from the straight line during the first few days is probably due to the increase in vitality as explained in the preceding paragraph.

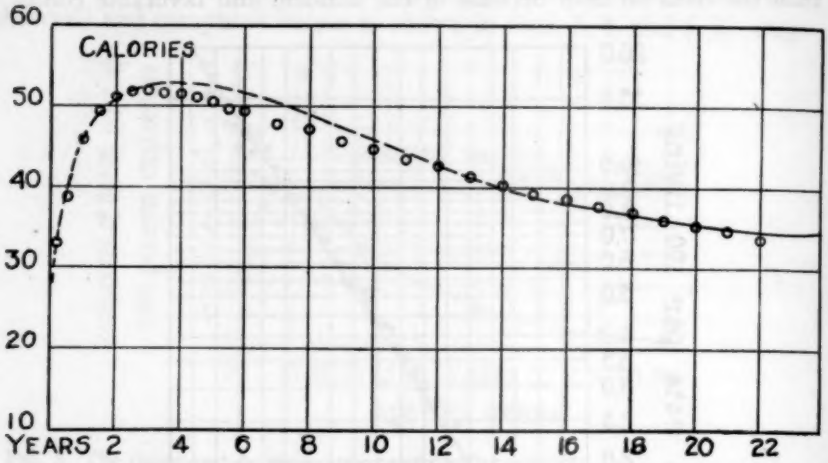

FIG. 7. "Variation of basal metabolism with age; calories per hour per square meter of body surface—Meeh's formula. Dash line shows average for males." Figure traced and legend quoted from Chart 2 of paper by Du Bois.[24] The circles represent the equations

$$y = 56.7e^{-0.024t} - 32e^{-1.224t}$$

if intrauterine period of growth is not considered;

$$y = 57.8e^{-0.024(t+0.78)} - 83e^{-1.224(t+0.78)}$$

if the intrauterine period of growth is considered, and if it is assumed that the duration of the intrauterine period of growth is 0.78 year.

An interesting if less satisfactory set of data from a statistical point of view is that published by Du Bois[24] on the course of basal metabolism with age expressed in terms of calories per square meter of body surface as determined by Meeh's formula. The agreement between observed and computed values as shown in Fig. 7 appears to be satisfactory considering the paucity of the data and the magnitude of experimental errors involved in the data.

[24] Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 887.

SUMMARY.

The course of decline of vitality with age due to the process of senescence, when not complicated by the process of growth, follows a simple exponential law; that is the degree of vitality or of senescence (defining vitality as the reciprocal of senescence) at any moment is, regardless of age, a constant percentage of the degree of vitality or senescence of the preceding moment. This exponential law is the same as the law of monomolecular change in chemistry.

During the actively growing period of life the index of vitality rises, due to the process of growth and the course of vitality in the case when the growing period is included in the vitality curve, follows a rising and falling course. This rising and falling course may often be represented by an equation containing two exponential terms which is practically the equation used to represent the course of accumulation and disappearance of a substance as the result of two simultaneous consecutive monomolecular chemical reactions.

ISOELECTRIC POINTS FOR THE MYCELIUM OF FUNGI.

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In a previous paper by the writer (13) evidence was presented which indicated that potato tuber tissue under the conditions of the experiments performed responded in water absorption, in the absorption and retention of dyes and in its effect upon the hydrogen ion concentration of dilute buffer mixtures much as an ampholyte with an isoelectric point in the vicinity of pH 6.0 In the present paper evidence is presented which indicates that the mycelia of *Rhizopus nigricans* and of *Fusarium lycopersici* respond in the absorption and retention of dyes much as amphoteric colloids with isoelectric points in the vicinity of pH 5.0 and 5.5, respectively; and that these points are of physiological importance in the growth of the two fungi.

An amphoteric compound like a protein reacts with and retains acid dyes on the acid side of the isoelectric point while it reacts with and retains basic dyes on the alkaline side of the isoelectric point. By determining, therefore, the reactions at which an acid dye like eosin is retained by the protein and at what reactions a basic dye like methylene blue or safranine is retained information may be gained as to the isoelectric point of the protein. This method of determining the isoelectric point has been used by Loeb (9) for gelatin and by Thomas and Kelly (17) for hide powder. Using the mycelium of the two fungi mentioned an attempt was made to determine the relation between hydrogen ion concentration and the response to dyes.

The mycelium of *Rhizopus nigricans* used in the experiments was grown on potato dextrose agar in test-tubes 2.5 × 20.5 cm. After several days growth a mycelial mat was formed which could be stripped off the agar. This mat was cut into pieces about 1.5 to

2.0 cm. square and used in the experiments. Tufts of the aerial mycelium were also used in some cases. The acid dyes used were water-soluble eosin, Martius yellow, acid fuchsin, and orange G. The basic dyes were methylene blue, basic fuchsin, and safranine.

The general procedure used was to stain the tufts of mycelium or pieces of mycelial mat for a few seconds or a few minutes with the dye and then wash the stained mycelium with buffer mixtures of different hydrogen ion concentrations. The buffer mixtures used consisted of mixtures of 0.1 M phosphoric acid and 0.1 M sodium hydroxide. During the washing the loss of the dye from the mycelium was observed and those reactions noted at which it was retained and at which it was lost.

The results with the dyes indicated that the mycelium of *Rhizopus nigricans* responded to both basic and acid dyes much like a protein with an isoelectric point in the vicinity of pH 5.0. The following are typical experiments.

Experiment 1.—Mycelial mat and tufts of mycelium were stained a few minutes with 1 per cent eosin and washed in buffer mixtures of 0.1 M phosphoric acid and sodium hydroxide of pH 3.5, 3.9, 4.5, 5.7, 5.8, and 6.9. After about 1 hour the mycelial mat at pH 3.5, 3.9, and 4.5 was bright red; from pH 5.7, was intermediate red; from pH 5.8, faint pink; and pH 6.9, white. After 24 hours the mycelium washed with buffer mixtures of pH 3.5 and 3.9 was bright red; pH 4.5, intermediate; 5.7 and 5.8, faint pink; and 6.9, white. While the mycelium washed with buffer mixtures of pH 5.7 and 5.8 retained a little of the eosin even with 24 hours washing the response was much like a protein with an isoelectric point between pH 4.5 and 5.7.

Experiment 2.—Repeated the first experiment using buffer mixtures of pH 3.4, 3.5, 3.7, 3.9, and 5.1. The mycelium washed with the first four buffer mixtures remained very red but lost the color in the last. From Experiments 1 and 2 the hypothetical isoelectric point would be located between pH 4.5 and 5.1.

Experiment 3.—The mycelium was stained with 1 per cent safranine and washed with the same buffer mixtures as in Experiment 2. After washing for 1 hour the mycelium at pH 3.4 was white; pH 3.5, 3.7, and 3.9, red; but much less red than at pH 5.1. After 48 hours washing the mycelium at pH 3.4 was white; at pH 3.5, 3.7, and 3.9,

very pale pink; and at pH 5.1, deep pink. While some of the basic dye was retained at pH 3.5 to 3.9 the response was much like a protein with isoelectric point between pH 3.9 and 5.1.

Experiment 4.—Used 1 per cent safranine and buffer mixtures of pH 3.9, 4.4, 4.6, 4.8, and 5.1. Color came out most rapidly at pH 3.9, considerably in 4.4, 4.6, and 4.8, very little in 5.1 as compared to 4.8. After about 30 minutes the mycelium at pH 3.9, 4.4, 4.6, and 4.8 was much lighter red than in 5.1. This was after several changes of the buffer mixtures.

Experiment 5.—Repeated Experiment 4 with eosin. After 30 minutes little difference noted. After 2 days the one in 5.1 was faint pink, others red. This would place the isoelectric point between 4.8 and 5.1 as determined by eosin and safranine. New growth from each piece indicated that the mycelium was not entirely dead.

Experiment 6.—Mycelial mat stained first with 1 per cent eosin and then 1 per cent methylene blue. Sum total a bluish purple. When washed in buffer mixtures of pH 3.5, 3.9, 5.1, 6.0, 6.7, the methylene blue came out at the acid end leaving the mycelium red at pH 3.5 and 3.9. The eosin came out at the alkaline end leaving mycelium blue at pH 6.0 and 6.7. At 5.1 both dyes were retained to some degree and at the end of washing the mycelium was purple.

Experiment 7.—Mycelial mat stained for 10 seconds in 0.05 per cent basic fuchsin and washed in buffer mixtures of pH 4.1, 4.5, 5.0, 5.5, 5.9, 6.5, and 7.2. After 50 minutes and three changes of the buffer mixtures the mycelium in pH 4.1, 4.5, and 5.0 was distinctly lighter in color than the balance. After 18 hours the mycelium in the first three most acid buffer mixtures was light brownish red and in pH 5.5 to 7.2, red. The difference between the color of the mycelium at pH 5.0 and that at 5.5 was marked.

Orange G, Martius yellow, and acid fuchsin were unsatisfactory. The first two because they washed out of the mycelium too readily. The last because it acts as an indicator and becomes colorless in the alkaline solutions.

The experiments on dye absorption and retention described above indicated that the mycelium of *Rhizopus nigricans* responded much as a protein would with isoelectric point between pH 4.8 and 5.1. The acid dyes were strongly held in the buffer mixtures of pH 4.8 or less

and the basic dyes used were strongly held at pH 5.1 or more. The response was not so sharp as can be secured with a protein like gelatin, a small amount of the acid dyes being held at reactions more acid than pH 5.1 even after comparatively long periods of washing.

If the mycelium of *Rhizopus nigricans* responds like a protein with an isoelectric point near pH 5.0 in dye absorption and retention we should also expect that its capacity for water absorption would be reduced at that point. The reduction in water absorption should result in a decreased turgor and a reduction in growth in the vicinity of pH 5.0 as contrasted to somewhat more acid and alkaline reactions. In order to test this hypothesis the mold was grown upon potato dextrose agar and potato dextrose broth the reactions of which were adjusted by means of phosphoric acid. On both media a reduction in growth was found at an initial reaction of pH 5.2. The experiments were performed as follows:

Potato dextrose agar was prepared by boiling 200 gm. of peeled and diced potatoes in 1 liter of tap water until soft. After filtering through cheese cloth 20 gm. of agar in shreds and 10 gm. of glucose were added. The original Sörensen value of the agar was 8.0. This figure was secured with three lots of potato dextrose agar and is higher than that reported by Hopkins (8). It was separated into lots of approximately 100 cc. and 1.0, 3.5, 5.6, 6.5, 7.0, 7.5, 8.0, 10.0, or 13.0 cc. of 0.1 M phosphoric acid were added to each lot. After sterilization the agar was poured into Petri dishes and the acidity of a portion measured colorimetrically by Gillespie's method. The acidities found are given in Table I. To the two most acid lots an additional 0.5 gm. of agar was added making them 2.5 per cent agar in order to make sure that they would gel. Four plates of each reaction were poured and inoculated in the center by means of a platinum needle. The spores of the inoculum made a streak 2 to 3 mm. long. Incubation was carried out in the dark at room temperature, approximately 25°C., and at the end of 24, 41, and 48 hours the diameters of the colonies were measured. The majority of the colonies were not circular. The longer and shorter diameters were therefore measured in each case. This made two measurements for each culture and eight for the four cultures at each acidity. The eight measurements were then averaged and are included in

Table I, and in the curves of Fig. I. The extent of the growth was least in the most acid plates, original pH 4.1. It increased to a maximum at pH 4.7 to 4.9, declined to minimum at pH 5.2; rose to a second maximum at pH 5.4, and then declined in the most alkaline media. These results agree fairly well with what might be anticipated from the action of the mycelium toward the absorption of dyes. It was found there that the mycelium of *Rhizopus nigricans* responded much like a protein with an isoelectric point in the vicinity

TABLE I.

Average Diameters of Colonies of Rhizopus nigricans at 24, 41, and 48 Hours on Potato Dextrose Agar of Different Acidities. Variations in Acidity Produced by the Addition of Phosphoric Acid.

Initial pH.	Average of diameters of colonies at		
	24 hrs.	41 hrs.	48 hrs.
	mm.	mm.	mm.
4.1	14.2	35.3	41.4
4.4	18.0	47.2	55.1
4.6	19.8	54.7	64.7
4.7	20.5	56.1	65.3
4.9	21.1	55.1	66.1
5.2	18.9	53.2	62.7
5.3	20.0	55.7	64.7
5.4	21.8	57.1	67.3
5.7	18.7	52.2	61.8
6.1	16.35	45.9	56.1

of pH 5.0. This would be, therefore, a point of low water absorption and a low point in growth due to the decreased water absorption. The growth curves show a low point at pH 5.2 between two maxima. This figure represents the initial pH of the potato dextrose agar.

What the hydrogen ion concentration was at the time the measurements of colony diameters were made was not determined. It was, without doubt, somewhat more acid. *Rhizopus nigricans* makes this medium more acid during its growth due very probably to the production of an organic acid. 2 days after the last measurements of growth were completed and the mycelium had completely covered the surface of the agar the mycelium and agar were removed from a few plates, melted, and the actual acidity determined. The agar of

original pH 6.1 had changed to pH 3.6 and that of original pH 4.1 and 4.7 had changed to a point below pH 3.0, too acid to be read by the colorimetric method used. The actual acidity affecting the mycelium in its growth was therefore greater than the initial Sörensen values would indicate. The mycelium advancing over fresh medium

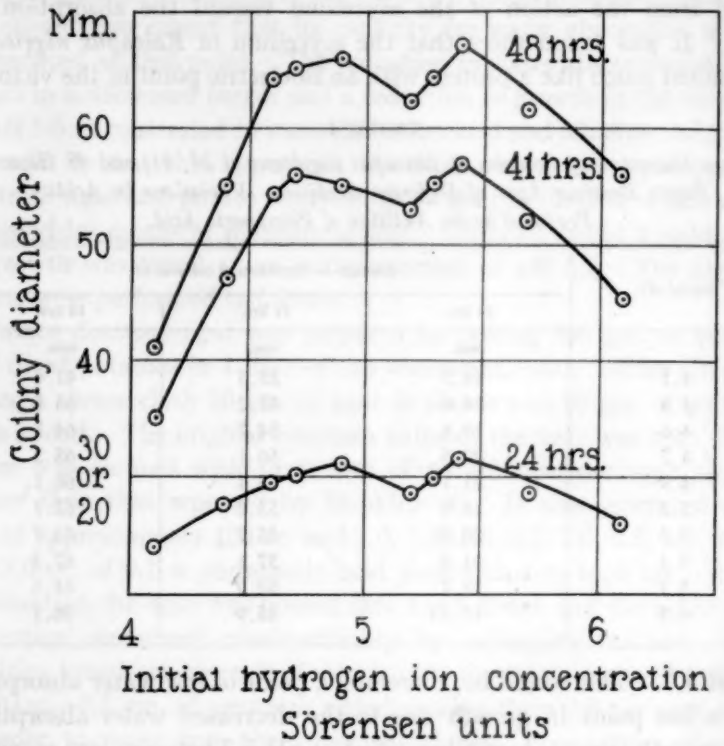

FIG. 1. Diameter of colonies of *Rhizopus nigricans* on potato dextrose agar at the end of 24, 41, and 48 hours plotted against the initial hydrogen ion concentration of the agar. Variations in reaction produced by the addition of phosphoric acid.

on the agar plates experiences, however, more nearly the action of the original acidity than is true in the liquid cultures reported below.

In order to substantiate the results secured with the agar an experiment with a liquid medium of similar constitution was performed. In this case a potato dextrose broth was prepared as previously but

no agar was added. The broth was heated at 15 pounds pressure for 20 minutes to complete coagulation and filtered. After filtration it was separated into lots of approximately 200 cc., and 2, 7, 10, 11, 12, 13, 15, or 20 cc. of 0.1 M phosphoric acid were added to each lot. 50 cc. of each lot of broth were placed in each of four 150 cc. Erlenmeyer flasks of Pyrex glass and the flasks were sterilized at 10 pounds pressure for 20 minutes. After sterilization three flasks of each acidity were inoculated with the spores of *Rhizopus nigricans*. The fourth flask was used to determine the actual acidity of the solution. The flasks were incubated in the dark at room temperature. After 52 hours 5 cc. of concentrated hydrochloric acid were added to each

TABLE II.

Dry Weight of the Mycelium of Rhizopus nigricans Produced in Potato Dextrose Broth of Different Acidities in 52 Hours. Variations in Acidities Produced by the Addition of Phosphoric Acid.

Initial pH.	Final pH.	Dry weight of mycelium.			
		1	2	3	Average.
		mg.	*mg.*	*mg.*	*mg.*
4.2	3.7	45.0	41.0	39.8	41.9±0.8
4.5	4.05	53.2	48.8	50.2	50.7±0.7
4.7	4.5	40.8	51.6	33.2	41.9±3.0
5.0	4.4	45.6	50.8	44.6	47.0±0.3
5.2	4.4	35.6	44.0	32.8	37.5±1.9
5.3	4.4	46.4	39.4	43.4	43.1±1.0
5.6	4.5	49.0	41.4	43.8	44.7±1.2
6.6	4.5	44.8	52.0	49.2	48.7±1.1

flask to stop the growth of the fungus. The mycelium was filtered into Gooch crucibles, dried at 110°C., and weighed. The initial Sörensen values of the solutions and the dry weight of the mycelium are given in Table II. In Fig. 2 the average dry weight of the triplicate cultures is plotted against the initial Sörensen values of the solutions. The dry weight increased from that of the most acid solution pH 4.2 to a maximum at pH 4.5, it then decreased to a minimum at pH 5.2 rising to a second maximum at pH 6.6. One point, that for initial pH 4.7 is decidedly off the curve. The dry weight, however, for this point also shows the greatest probable error. The results found here agree with the growth experiment

on the agar. A low point between two maxima was found at initial pH 5.2. The average acidity for this culture solution which probably represents more nearly the actual acidity experienced by the fungus was pH 4.9.

Another method for determining the isoelectric points of ampholytes is that described by Michaelis (10). If the ampholyte is placed in dilute buffer mixtures the reactions of the buffer mixtures become more acid on the alkaline side of the isoelectric point due to the

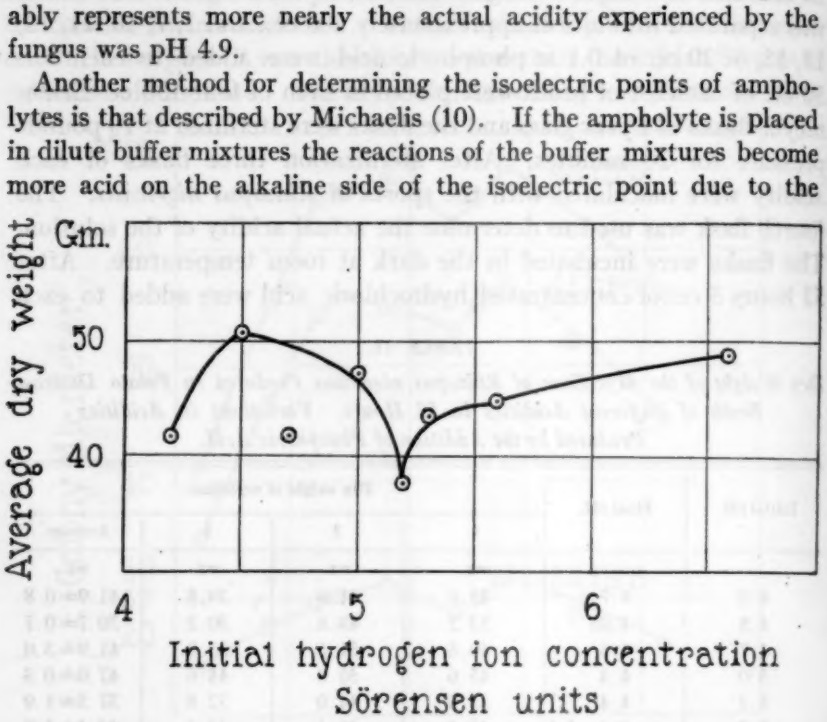

FIG. 2. Dry weight of mycelium of *Rhizopus nigricans* grown in potato dextrose broth plotted against the initial hydrogen ion concentration of the broth. Variations in reaction produced by the addition of phosphoric acid.

reaction between cations and the ampholyte. On the acid side of the isoelectric point the shift in the reaction is toward greater alkalinity due to reaction between anions and the ampholyte.

Experiments of this type were performed with the mycelium of *Rhizopus nigricans*. Buffer mixtures of 0.01 M phosphoric acid and 0.01 M sodium hydroxide were used. A piece of the mycelial mat was placed in approximately 50 cc. of the solution and at intervals 5 cc. were removed and the hydrogen ion concentration determined. The data are summarized in Table III. In the first 4 hours of contact the buffer mixtures of pH 4.1 and 4.4 alone show much change in

reaction. After 19½ hours the buffer mixture of pH 5.2 shows a decided change toward greater acidity. A repetition of this experiment using buffer mixtures of 4.5, 5.2, 5.4, 5.6, and 6.2 showed in 18 hours the following pH values: 5.6, 5.7, 5.7, 5.6, and 6.4.

The mycelium of *Rhizopus nigricans* apparently did not affect buffer mixtures of dilute phosphoric acid and sodium hydroxide as an ampholyte with an isoelectric point in the vicinity of pH 5.0 would be expected to do. The change in reaction toward greater acidity on the alkaline side of pH 5.0 was not evident. The change in reaction in buffer mixtures more acid than pH 5.0 was greater than would be expected.

TABLE III.

Effect of the Mycelium of Rhizopus nigricans upon the Hydrogen Ion Concentration of Buffer Mixtures of 0.01 M *Phosphoric Acid and 0.01* M *Sodium Hydroxide.*

Original pH.	pH after				
	½ hr.	1 hr.	1½ hrs.	4 hrs.	19½ hrs.
4.1	4.2	4.3	4.4	4.6	5.2
4.4	4.5	4.7	4.9	5.1	5.3
5.2	5.3	5.2	5.2	5.2	5.8
5.4	5.4	5.3	5.2	5.3	5.4
5.6	5.6	5.6	5.5	5.6	5.6
5.9	5.9	5.9	5.9	5.9	5.9
6.3	6.2	6.2	6.1	6.1	5.9

Scott (16) has grown *Fusarium lycopersici* in culture solutions of varying acidities. When the dry weights of the mycelium were plotted against the Sörensen value of the solutions a curve was produced with a minimum between two maxima at average pH about 5.5. If we are correct in our assumptions we should find that the mycelium of this fungus responds to dyes much like a protein with an isoelectric point at pH 5.5. Experiments similar to those carried out with the mycelium of *Rhizopus nigricans* indicated that the mycelium of *Fusarium lycopersici* does respond in dye absorption much like a protein with an isoelectric point near pH 5.5.

The mycelium was grown for the writer by Scott on a culture solution. The dyes used were Martius yellow, acid fuchsin, eosin, methylene blue, and basic fuchsin. The buffer mixtures used were made

from 0.1 M phosphoric acid and 0.1 M sodium hydroxide. The best results were secured with basic fuchsin, eosin, and a combination of eosin and methylene blue.

Experiment 1.—The mycelium of *Fusarium lycopersici* was stained with 1 per cent eosin for a few minutes and washed in buffer mixtures of 0.1 M phosphoric acid and 0.1 M sodium hydroxide. The buffer mixtures used were of pH 3.5, 3.9, 5.3, 6.0, 6.7, and 7.3. After 18 hours the mycelium from pH 3.5 and 3.9 was bright red; from 5.3, deep pink; from 6.0, light pink; and from 6.7 and 7.3, almost white. The distinct difference in color between the mycelium from the buffer mixture of pH 5.3 and that of pH 6.0 would suggest an isoelectric point between pH 5.3 and 6.0.

Experiment 2.—Mycelium of *Fusarium lycopersici* stained for 10 seconds in 0.05 per cent basic fuchsin and treated with buffer mixtures of pH 4.1, 4.5, 5.0, and 5.5, was distinctly lighter in color than that from buffer mixtures of pH 5.9 and 6.5. The mycelium in pH 7.2 was also light in color. After 18 hours washing the mycelium in pH 4.1 was almost white, that in pH 4.5 showed faint color, that in pH 5.0 and 5.5 showed a little more color, but much less than the mycelium in pH 5.9 and 6.5. The mycelium in pH 7.2 was also faint in color but darker than that from the acid buffer mixtures. This would place the isoelectric point between pH 5.5 and 5.9.

Experiment 3.—Mycelium stained with 1 per cent eosin for a few minutes and then a few seconds with 0.1 per cent methylene blue. Mycelium a deep purple. Placed to wash in buffer mixtures used in Experiment 2. After 24 hours washing the mycelium at pH 4.1 was deep red; at pH 4.5 and 5.0, red with little blue on edges; at 5.5, pink; at pH 5.9, 6.0, and 7.2, almost white. On exposure to the air blue color developed in the mycelium from the three most alkaline solutions and the final appearance was as follows: pH 4.1, 4.5, 5.0, red; 5.5, faint pink, little blue; 5.9, 6.5, 7.2, blue. This would place the isoelectric point near pH 5.5. In a similar experiment when the mycelium was stained with 1 per cent eosin and 1 per cent methylene blue, it was found impossible to remove all the methylene blue from the mycelium in the acid buffer mixtures even after 48 hours washing. The relative predominance of the red or blue color, however, substantiated the results where less intense staining was used.

The same difficulties with Martius yellow and acid fuchsin found in the case of *Rhizopus nigricans* were also found with *Fusarium lycopersici*.

DISCUSSION.

How far the analogy between the responses of plant tissue and those of an amphoteric colloid, with a definite isoelectric point can be pushed is a question of fundamental importance.

The effect of acid and alkaline reactions on the absorption or loss of dyes by plant cells is evidence which supports such an analogy. Several investigators have observed the favorable effect of acid reaction upon the uptake of acid dyes and its inhibitory effect upon the absorption of basic dyes, by living or dead plant or animal cells and *vice versa*, the favorable effect of basic reaction on the absorption of basic dyes and its inhibitory effect upon the absorption of acid dyes. Robertson (14) observed it working with 0.01 N hydrochloric acid and 0.01 N sodium hydroxide upon the staining of fat cells, connective tissue cells, and red blood corpuscles by acid and basic dyes. Harvey (6) found that basic dyes as a rule could not enter cells in the presence of a trace of acid in the medium whereas certain acid dyes did not enter cells in neutral or weakly alkaline solution but readily stained and killed the cells in weakly acid solution. He explained this as due to an effect of the acidity or alkalinity upon the dissociation of the dye. Endler (4), Bethe (1, 2), Rohde (15), and Collander (3) extended and confirmed Harvey's observations but explained the results as due to an effect of the acidity or alkalinity upon the power of the cell colloids to absorb the dyes.

The effect of acidity and alkalinity upon the conductivity of *Laminaria* tissue observed by Osterhout (11, 12) is analogous to their action upon the conductivity of an amphoteric colloid.

The production of a double maximum curve under conditions of different acidities and alkalinities for the absorption of water by plant tissue, for the germination of seeds or spores, for the growth of plants, and for the infection of plants by certain fungi offers additional evidence in the same direction. Attention was called to this in an earlier paper (13).

Endler (4) found that the order in which certain salts affected the absorption of methylene blue by *Elodea* cells in alkaline solution was reversed in acid solution. He concluded that the protoplasm of *Elodea* acted like an amphoteric colloid with an isoelectric point between pH 3.8 and 4.1. The death of the cell changed the isoelectric point to a more alkaline position.

Falk and Shaugnessy (5) state that the bacteria act like amphoteric colloids with two isoelectric points. Hoagland and Davis (7) found that the nitrate ion was absorbed by *Nitella* cells from solutions of pH 5.0, 5.2, or 6.2 but very slightly from solutions of pH 7.0, 7.2, 8.5, or 9.0+. This is analogous to the action of an ampholyte with isoelectric point between pH 6.2 and 7.0.

Difficulties in the application of such a conception to the living cell particularly the plant cell are numerous. The complexity and diverse make-up of the cell contents and the presence of a large and aqueous central vacuole in the majority of plant cells are two of the most obvious. Nevertheless the positive evidence at hand indicates that the conception of the living cell as an amphoteric colloid is worthy of serious consideration.

SUMMARY.

1. Mycelium of *Rhizopus nigricans* when stained with certain acid and basic dyes and washed with buffer mixtures of 0.1 M phosphoric acid and sodium hydroxide responded much like an amphoteric colloid with an isoelectric point near pH 5.0.

2. When grown on potato dextrose agar the reaction of which was varied with phosphoric acid the extent of colony growth of *Rhizopus nigricans* plotted against the initial Sörensen value of the agar produced a double maximum curve with the minimum between the two maxima at initial pH 5.2.

3. When grown in potato dextrose broth the reaction of which was varied with phosphoric acid the dry matter produced by *Rhizopus nigricans* plotted against the Sörensen value of the broth produced a double maximum curve with the minimum between the two maxima at initial pH 5.2 or average pH 4.9.

4. Mycelium of *Rhizopus nigricans* placed in buffer mixtures of 0.01 M phosphoric acid and sodium hydroxide of pH 4.1 to 6.3, changed the reaction in most cases toward greater alkalinity.

5. Mycelium of *Fusarium lycopersici* stained with certain acid and basic dyes and washed with buffer mixtures of 0.1 M phosphoric acid and sodium hydroxide responded much like an amphoteric colloid with an isoelectric point near pH 5.5.

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LUMINESCENCE IN PELAGIA NOCTILUCA.

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Pelagia noctiluca is a medusa found in the Mediterranean during the spring months. This animal shows a diurnal rhythm in its luminescence. Early in the evening it begins to give brillant light in response to mechanical stimulation. If the animal is weakly stimulated, as by a mere touch, the area of luminescence is confined to the region stimulated (local luminescence); but if the stroke is a vigorous one, the whole bell and tentacles respond with a glow which may last for some seconds (general luminescence) (1).

Rhythmical Pulsations and General Luminescence.

As in the case of other medusæ, *Pelagia* pulsates rhythmically, and the rate of the rhythm is quickened by stimulation. The phenomena of general luminescence and pulsation, as will be shown, are both under the control of the nervous system and in each case the reaction depends upon the ionic composition of the fluid in which the animal is immersed. As to the rhythmical contractions of medusæ, numerous studies have been made showing the dependence of contractions upon the ionic content of the surrounding fluid: Loeb on *Gonionemus* (2) and *Polyorchis* (3), Herbst on *Obelia* (4), Mayer on *Cassiopeia* (5), Bethe on *Rhizostoma* (6). If *Pelagia* be placed in artificial sea water (van't Hoff's solution) containing solutions of pure salts isosmotic with the sea water of Naples Bay (0.6 M) in the following proportions, NaCl 100, $CaCl_2$ 1.5, KCl 2.2, $MgCl_2$ 7.8, $MgSO_4$ 3.8 plus 20 cg. $NaHCO_3$ per 1,000 cc. of solution, the medusa lives and reacts just as in ordinary sea water. But if one of the cations is omitted from the solution the conduct of the animal with regard to pulsations and luminescence is entirely altered.

1. In a solution from which $CaCl_2$ is omitted the pulsations of *Pelagia* stop in 3 to 5 minutes. At the same time general luminescence fails, so that when the animal is strongly stimulated light appears only in the area of contact. If now the proper amount of $CaCl_2$ be added to the solution or if the animal be returned to artificial sea water, the beats reappear within 90 seconds and simultaneously the power of general luminescence is restored. It must, therefore, be concluded that $CaCl_2$ is necessary to the conduction of impulses both for the muscular beats and for general luminescence.

2. If the solution lacks K ions, after 10 minutes the pulsations stop in the diastolic phase and at the same time the power of general luminescence is lost. When the animal is replaced in sea water or in complete van't Hoff's solution the beats return within 65 seconds and general luminescence is reestablished in 140 seconds. These results prove that K like Ca is necessary for normal beats and for the conduction of the impulse for general luminescence.

3. In a solution containing no Mg salts, *Pelagia* shows great acceleration of beat, and after 11 minutes stops in systole with spasmodic fibrillation. During this time automatic flashes of light appear and, if the body is touched, the whole surface breaks into light and glows for some seconds. This condition persisted for at least an hour and a quarter, at the end of which time the observations were discontinued. Since the absence of Mg results in a condition of hyperirritability both with regard to rhythmical contractions and general luminescence, it follows that Mg ions must act to decrease irritability. The locus of the action of Mg on the luminescence reaction is some part of the nervous system, since an excess of Mg inhibits general luminescence, but does not affect local luminescence.

Inhibition of Luminescence by Light.

The fact of nightly periodicity in the luminescence of *Pelagia* suggests the possibility of inhibiting luminescence at night by means of artificial illumination. Such a relation has been shown to exist in other forms, (Allman (7), Peters (8), Heinemann (9)). It was found that exposure to light of the carbon arc resulted in inhibition of general luminescence, but did not affect local luminescence. These results suggest that strong illumination acts on the nervous mechan-

ism to inhibit general luminescence, but exercises no direct effect on the luminescent organs themselves, since local luminescence persists. It should be noted, however, that during the day excitation of *Pelagia* fails to elicit local luminescence even after 5 hours in the dark room.

It was next attempted to determine whether inhibition of general luminescence by light depended upon the absolute intensity of the light alone or upon the quantity of light (intensity time). Two series of experiments were completed with different individuals. The animal was put into a rectangular glass box filled with sea water, and the preparation placed in such a position that it lay directly in the path of the rays of a carbon arc of 1,000 c.p. The specimen was always tested for general luminescence before being subjected to illumination. If found to be properly sensitive, light from the arc

TABLE I.

Intensities.	A Time.	B Time.
meter candles	*sec.*	*sec.*
4,000	22	180
1,000	52	480
250	150	720
62.5	600	

was admitted by removing a screen of black cloth. After an exposure for a certain number of seconds the arc was extinguished and the animal tested for general luminescence. If local responses alone were obtainable then it was considered that sufficient illumination had been given, and another trial with a shorter exposure was next made, but only after the specimen had been allowed to recover its power of general luminescence by remaining in the dark; this usually took 6 to 10 minutes. The table gives approximate values for the times of exposure which are required at the intensities stated to suppress general luminescence. The data obtainable were not sufficiently extensive to warrant further mathematical treatment.

The figures show that inhibition of general luminescence by light may be regarded as a function of the quantity of light received by the organism, and that individual *Pelagia* differ in their sensitivity to light.

Reaction of Luminescent Material.

In the course of the experiments it was found that at night *Pelagia* threw off quantities of luminescent material (1) which stuck to hands or towel and which would glow brightly if rubbed or if washed with fresh water. Use was made of this fact to prepare an indicator paper. This was done by rolling a *Pelagia* on filter paper, which was, as a result, saturated with luminescent material and if kept moist would continue to react for an hour or longer. Normally this indicator paper showed no light, but when torn, rubbed or put into proper solutions it glowed with light.[1]

TABLE II.

$MgSO_4$	18	16	14	12	10	8	6	4	4	2					
$MgCl_2$	2	4	6	8	10	12	14	16	16	18					
Result	S	S	S	S	W	W	W	W	0	0					
$BaCl_2$	10	8	6	4	2	$SrCl_2$	10	8	6	4	2				
$MgCl_2$	10	12	14	16	18	$MgCl_2$	10	12	14	16	18				
Result	W	W	W	0	0	Result	W	0	0	0	0				
KCl	10	8	6	4	2	KCl	10	8	6	4	2				
$MgCl_2$	10	12	14	16	18	NaCl	10	12	14	16	18				
Result	M	M	W	W	0	Result	W	W	W	0	0				

Letters indicate comparative intensities of light evoked. W = weak, M = medium, S = strong, 0 = no light.

When a piece of luminescent test paper was put into sea water no lasting glow was obtained, nor did the paper show luminescence when put into pure cane-sugar solution of concentration 1.1 M (isosmotic with the sea water of Naples Bay). This fact suggests two things: (1) that where the ions are balanced the luminescence reaction does not occur, and (2) in the absence of ions there is no reaction. Pure solutions of certain salts, however, in concentrations isosmotic with sea water, namely 0.6 M, caused the indicator paper to glow brightly for several seconds or even minutes. At the outset, we found that solutions of pure salts were effective in activating the paper in the following order as to brightness, $MgSO_4$, K_2SO_4, Na_3 citrate, KCl,

[1] The Ctenophores, *Eucharis multicornus* and *Beroë ovata*, and the Anthozoan, *Pennatula phosphorea*, yield material which behaves similarly.

$BaCl_2$, $SrCl_2$, $CaCl_2$, and LiCl while NaCl and $MgCl_2$ caused no luminescence of the test paper. It can further be shown that $MgCl_2$ and NaCl act as inhibitors to the reaction since if present in sufficient quantity they suppress the positive action of $MgSO_4$, KCl, $BaCl_2$ and $SrCl_2$. In the following experiments the solutions were made up to 20 cc. and the relative quantities of the two salts altered as indicated in Table II.

The salt solutions used were approximately neutral in reaction, but it was not possible to determine their exact pH values on account of lack of facilities in the Naples laboratory. However, the matter has been checked by one of us, since his return to America, using the luminescent material from *Mnemiopsis*. This behaves similarly to the material obtained from *Pelagia*. It was found that salt solutions of pH 7.7 caused characteristic effects on the luminescent material. Luminescence of the indicator paper occurs in solutions of K_2SO_4, KCl, $MgSO_4$, $CaCl_2$, but does not take place in NaCl and $MgCl_2$. Alterations in the pH values of the salt solutions between pH 6.0 and 8.0 are without effect on the phenomenon.

Since NaCl and $MgCl_2$ are present in sea water in sufficient concentrations to inhibit the activity of KCl and $MgSO_4$, the luminescent material of *Pelagia* does not glow in sea water.

Alkalies of concentration N/10 made up in isosmotic cane-sugar solution caused luminescence while mineral acids N/10 inhibited the action of $MgSO_4$ and KCl (10). Ammonia in concentration 0.027 N to 0.9 N made up in sucrose solution 1.1 M caused strong luminescence for 10 minutes at 20°.

In order to gain further information on the nature of the luminescence reaction, we determined the temperature coefficients for the reaction in $MgSO_4$ and in ammonia 0.9 N. The procedure consisted in bringing the solution to the required temperature in a water bath, then a piece of indicator paper was introduced into the solution and the time between the introduction of the paper, and the cessation of light was measured with a stop-watch. Experiments 1 and 2 were done with $MgSO_4$ solution 0.6 M and Experiment 3 with ammonia in sugar solution, the sugar solution being of concentration 1.1 M, and the ammonia 0.9 M.

TABLE III.

Experiment 1.			Experiment 2.			Experiment 3.		
Temperature of solution.	Duration of light.	Temperature coefficient.	Temperature of solution.	Duration of light.	Temperature coefficient.	Temperature of solution.	Duration of light.	Temperature coefficient.
°C.	sec.	Q_{10}	°C.	sec.	Q_{10}	°C.	sec.	Q_{10}
5	305	1.6	8	108	1.8	10	640	1.1
15	188		18	65	1.4	20	593	3.7
20	121	1.8	28	42	2.6	30	160	2.0
25	105	5.0	38	16	1.2	40	81	2.3
30	24	2.8	48	13		50	35	(9.0)*
35	37	1.4				60	4	
40	17							
47	6							
Average.....		2.52	Average.....		1.75	Average.....		2.27

* Very little light given, the magnitude of the temperature coefficient shows the destructive action of high temperature to be predominant. This figure is therefore omitted from the calculations.

The temperature coefficient for the reaction is thus about 2 for a difference in temperature of 10°C. This conforms to the van't Hoff-Arrhenius rule for chemical reactions and suggests that in the case of luminescence we have to do with a chemical reaction. This conclusion is in harmony with the work of Amberson (11) who studied the decay curve of luciferin and found the luminescence reaction to follow the course of a monomolecular reaction.

Harvey, as a result of his work on luminous bacteria, suggests that luminescence and cytolysis may be related. In the case of *Pelagia* such a view seems plausible since water and hypotonic solutions cause the luminescent material to glow. But several facts serve to indicate that cytolysis and luminescence in *Pelagia*, are not conditioned by similar reactions.

(1) Raising the temperature of the sea water in which luminescent material is immersed does not cause the luminescent reaction although such a procedure does produce cytolysis of egg cells, blood corpuscles, etc.

(2) Solutions of pure salts in the concentration used to cause luminescence, do not produce cytolysis.

CONCLUSIONS.[2]

1. Ca and K condition the irritability of *Pelagia* both in regard to rhythmical contractions and general luminescence. If either ion is omitted from the solution conduction of stimuli for pulsations and luminescence does not occur, although local responses still persist.

2. When Mg is omitted from the solution, *Pelagia* shows hyperirritability with respect to rhythmical contraction and general luminescence. This is referable to the unantagonized action of K and Ca ions.

3. Exposure to the carbon arc suppresses general luminescence, the effect depending upon the quantity of light *i.e.* intensity × time of exposure.

4. The luminescent material secreted by *Pelagia* is inactive in sea water, but when put into salt solutions is activated by some of them. The efficiency of the salts, measured by brightness of light, is in the following order: $MgSO_4$, K_2SO_4, Na_3 citrate, KCl, $BaCl_2$, $SrCl_2$, $CaCl_2$, and LiCl while NaCl and $MgCl_2$ act as inhibitors.

5. Acidity inhibits the reaction, alkalinity promotes it. NH_4OH in concentrations 0.27 N to 0.9 N causes luminescence for 10 minutes at 20°.

6. The average temperature coefficient for the reaction of the luminescent substance when activated by ammonia or $MgSO_4$ is 2.18 for a temperature interval of 10°C.

[2] A number of the facts which we have determined regarding the luminescence of *Pelagia* incidentally have a bearing on the views put forth by Pierantoni (12) and Zirpolo (13) according to which luminescence in Metazoa is caused by symbiotic luminous bacteria (16). Such a view is untenable as regards *Pelagia* since (1) this animal repeatedly excretes within a very short time enormous quantities of luminous material, a result which can come only from a process akin to glandular secretion. (2) It is impossible that bacteria and living cells in general could continue to live and function for 10 minutes in 0.9 N solution of ammonia, yet the luminescent material of *Pelagia* glows brilliantly in such a solution for 10 minutes at 20°, and for 8 minutes in 0.1 N NaOH. In fact, E. N. Harvey (10) finds that a hydroxyl concentration of N/1,000 is sufficient to extinguish the light of luminescent bacteria in 10 minutes and E. B. Harvey (15) reports that the luminescence of *Noctiluca* is extinguished in 2 minutes by N/250 NaOH. (3) General luminescence in *Pelagia* is under the control of the nervous system whereas Harvey finds that luminous bacteria living symbiotically on fish, glow continuously and are not affected in this respect through the nervous system (14).

7. The luminescence reaction cannot be the result of cytolysis, because (*a*) raising the temperature of sea water in which luminous material is immersed does not cause luminescence, although sufficient to produce cytolysis. (*b*) The salt solutions used in our experiments to cause luminescence, do not act cytolytically on cells in general.

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THE NERVOUS MECHANISM OF COORDINATION IN THE CRINOID, ANTEDON ROSACEUS.

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In the crinoid, *Antedon rosaceus*, each of the five rays is branched into two arms, the point of bifurcation being 3 or 4 mm. from the central disk (Fig. 1). The branches or arms are invested with two rows of pinnules oppositely placed, thus giving to the animal its characteristically feathery appearance. On the dorsal or aboral side of the central disk are about twenty cirri by means of which

FIG. 1. Normal specimen of *Antedon* with arms extended in the resting position

the animal when at rest attaches itself to some inert object. Hence, *Antedon*, unlike the starfish and sea urchins, rests with the aboral side in contact with a surface, the oral face free. We shall use as synonymous the terms oral and ventral, aboral and dorsal.

Reaction of the Arms to Excitation.

The righting and swimming movements of *Antedon* are rapid and seemingly complex. Although one of these animals may remain in the same position for weeks if undisturbed, any strong stimulation causes the cirri to loosen their grasp, following which the animal swims through the water until another object is found to which the

cirri may be attached. Swimming is accomplished by alternate motions of the arms; the first phase of each movement is oral flexion followed by extension and an aboral stroke. Two arms of a given ray, move alternately so that while one is executing the aboral stroke its mate is flexing ventrally. If the animal is laid oral side down in the aquarium, righting is accomplished by vigorous ventral flexion of the arms.

In a resting specimen, mechanical stimulation of a single arm causes one of two types of reaction depending upon whether stimulation is weak or strong. Weak or moderate stimulation causes ventral flexion of the arm, strong stimulation as by pinching with the forceps, results in dorsal extension of the arm stimulated (*a* in Fig. 2). and

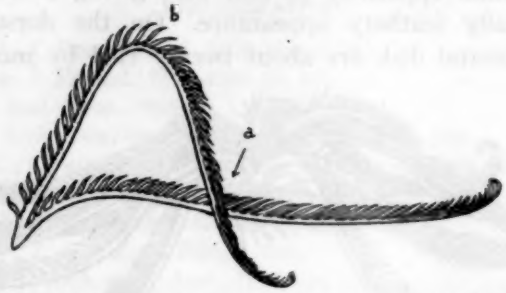

FIG. 2. Isolated ray consisting of arms *a* and *b*. As a result of strong stimulation of *a* that arm shows dorsal stroke while its mate, *b*, bends ventrally.

ventral flexion (*b* in Fig. 2) of the adjacent arms. This last reaction is followed by the swimming rhythm of the whole animal. In the case of a fatigued specimen, however, strong stimulation produces the same result as does weak stimulation of a normal individual. This is analogous to the situation in dogfish described by Maxwell (1) in which weak stimulation gave a positive and strong stimulation a negative stereotropic reaction when normal animals were employed, while in fatigued animals strong stimulation was followed by a positive reaction. Results of a similar character have been reported for the stereotropic reaction of the tube feet of *Asterias forbesii* (2), and by von Uexküll for the tension of the radial muscles of the spine of sea urchin (3). It seems apparent, therefore, that Maxwell's conclusion regarding the locus of the reversal mechanism is also cor-

rect for *Antedon*, namely that the character of the reaction is determined centrally and is not referable to the stimulation of different sets of sense organs, since strong stimulation acts upon the same sensory elements to produce opposite results depending upon the degree of fatigue in the animal.

Coordination Between the Arms of One Ray.

As has been stated, *Antedon* swims by means of alternate ventral flexion and dorsal extension of the arms. A single ray may be isolated and the reflexes of its two arms studied. Such preparations live and show vigorous reactions for hours after being cut away from the central disk. In a preparation of this kind (Fig. 2), if one arm be strongly stimulated (*a*), extension and dorsal stroke follow while

FIG. 3. Isolated ray which has been treated with strychnine. Stimulation of one arm results in both arms assuming this position of ventral flexion and retaining it for several minutes.

the paired arm (*b*) executes the opposite phase, namely, ventral flexion. In vigorous rays a series of rhythmical movements may follow, the phases of flexion and extension alternating in the two arms. This suggests reciprocal inhibition. It should be possible to test this hypothesis by the use of strychnine since strychnine has been shown to abolish inhibitions in other echinoderms (4). For this purpose an excised ray of *Antedon* was put into strychnine sulfate dissolved in sea water 1:10,000 and allowed to remain 10 minutes. At the end of this time the ray was returned to sea water and the reflexes tested. In all cases stimulation of one arm resulted in strong ventral flexion of both arms (Fig. 3). The extension phase did not occur, both arms remaining flexed for several minutes. In

other words reciprocal inhibition is abolished by strychnine, which proves that in *Antedon* the alternation of swimming movements is determined by a nervous mechanism similar to that present in the starfish arm. Nicotine in concentration 1:10,000 has an effect similar to strychnine (5). In this connection there are two exceptional features to be noted. First, strychnine acts on *Antedon* to produce a reaction similar to that brought on by weak stimulation in the normal animal, whereas the rule in other forms is that the strychnine spasm is similar to the reaction to maximal stimulation in the normal animal. Second, nicotine poisoning gives the same picture as does strychnine, although in the starfish the two alkaloids cause respectively ventral and dorsal spasms.

Coordination Between the Rays.

The crucial problem of locomotion in any animal is coordination of the muscular movements. It has been shown in the case of the starfish that coordination between the rays depends upon the fact that impulses arising from one ray travel around the circumoral nerve ring to the other rays and that an impulse decreases in effectiveness as it proceeds from its point of origin (6). This rule has been shown to hold for the annelid nerve cord (7). In order to determine whether coordination between the rays in *Antedon* might have a similar mechanism, the following experiments were carried out. If, in a normal *Antedon*, one arm be stimulated by pinching with the forceps, the arm stimulated reacts with strong dorsal bending and the other rays show varying degrees of reactivity, flexing ventrally and turning laterally toward the stimulating arm. In sensitive individuals the wave of excitation may affect all the rays, but the response is always most marked in those adjacent to the arm stimulated.

To make the point entirely clear, consider the case in which the dorsal nervous pentagon (8) (Figs. 4 and 5) which connects the rays, is interrupted at one point by an incision. Let the rays be numbered consecutively 1, 2, 3, 4, 5, and an incision be made between 1 and 5, thus breaking the nervous connection between the rays at that point, then stimulation produces the following series of reactions. Excitation of ray 1 causes strong reaction in 1 and 2, moderate reaction

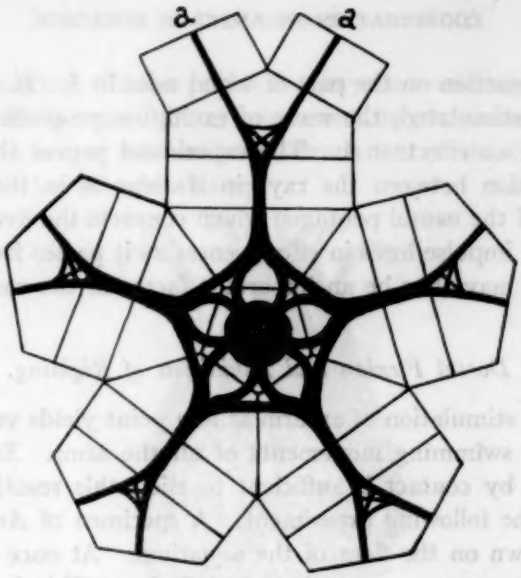

FIG. 4. Diagram (after Marshall) showing arrangement of dorsal nerve tracts of the centrum in a pentagon. Branches are shown going to each of the five rays, and further branching to each arm, *a*, *a* (axial cords).

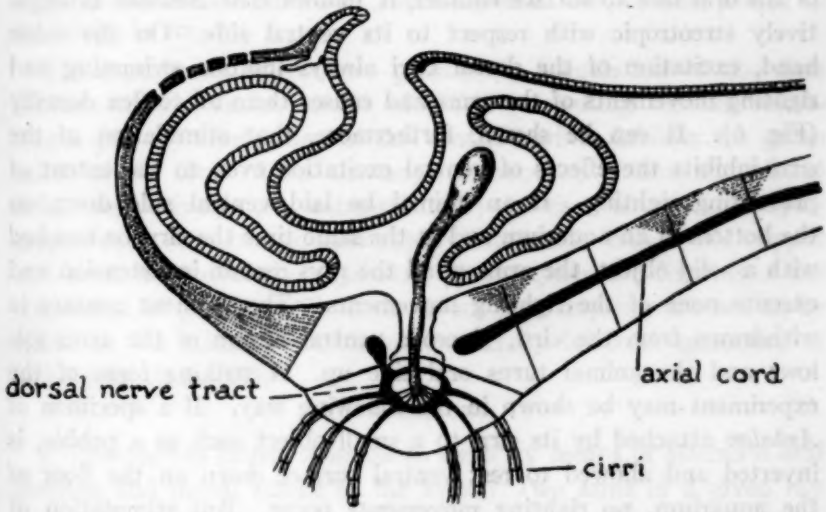

FIG. 5. Diagram (after Marshall) showing longitudinal section. Dorsal nerve tract of the centrum and distribution of branches to the cirri. Axial nerve cord of arm is shown.

in 3, weak reaction on the part of 4 and none in 5. If, on the other hand, 5 be stimulated, the wave of excitation proceeds from 4 to 3 to 2 having no effect on 1. The experiment proves that the basis of coordination between the rays in *Antedon* is in the conducting properties of the neural pentagon which connects the five rays. The fact that an impulse loses in effectiveness as it passes from the point of its origin may thus be an important factor in the coordination of movement.

Dorsal Flexion and Inhibition of Righting.

Moderate stimulation of an arm at any point yields ventral flexion followed by swimming movements of all the arms. Even stimulation caused by contact is sufficient to elicit this reaction. This is shown by the following experiment. A specimen of *Antedon* is put oral face down on the floor of the aquarium. At once all the arms make vigorous movements of ventral flexion. This is followed by active swimming, and as a result righting follows, *i.e.* the animal turns oral side up. Since the reaction just described is a reaction of the oral face to surface contact, it follows that *Antedon* is negatively streotropic with respect to its ventral side. On the other hand, excitation of the dorsal cirri always inhibits swimming and righting movements of the arms and causes them all to flex dorsally (Fig. 6). It can be shown, furthermore, that stimulation of the cirri inhibits the effects of ventral excitation even to the extent of preventing righting. If an animal be laid ventral side down on the bottom of an aquarium and at the same time the cirri be touched with a solid object, the arms of all the rays remain in extension and execute none of the righting movements. The moment contact is withdrawn from the cirri, vigorous ventral flexion of the arms follows and the animal turns oral side up. A striking form of the experiment may be shown in the following way. If a specimen of *Antedon* attached by its cirri to a small object such as a pebble, is inverted and allowed to rest ventral surface down on the floor of the aquarium, no righting movements occur. But stimulation of the oral surface caused by contact with the bottom forces the cirri to release their grasp on the pebble. The latter now lies loosely upon the cirri but still exerts enough excitation to inhibit any move-

ment of the arms. If the pebble is now removed from its contact with the cirri vigorous righting movements begin, since these movements are no longer inhibited by stimulation of the cirri, and the animal comes to rest oral side up.

FIG. 6. Aboral bending of all the arms in response to stimulation of the cirri. In the figure the pair of arms at the front are shown drawn apart in order to allow a view of the cirri and other arms. Such a reaction always follows picking up an *Antedon* by the cirri.

The experiment proves two points, (1) stimulation of the cirri inhibits ventral flexion to the extent of preventing righting movements and (2) stimulation of the ventral surface inhibits the grasp reflex of the cirri. Thus the tropistic impulses of oral and aboral sides, respectively, act antagonistically—a case of dynamic symmetry with reference to structurally dissimilar halves (*i.e.* upper and lower halves) of the organism. This experiment disposes of the contention of Jennings (9) to the effect that tropisms are not to be found in the echinoderms since structurally bilateral symmetry is lacking in them (10).

CONCLUSIONS.

1. Stimulation causes *Antedon* to swim by means of alternate oral bending and dorsal stroke of the arms. Two arms of a given ray move alternately so that while one is executing the aboral stroke its mate is flexing ventrally. This implies reciprocal inhibition.

2. Recriprocal inhibition between the two arms of an isolated ray can be abolished by the use of either strychnine or nicotine.

3. Coordination between the rays is referable to the conducting properties of the nervous pentagon which connects the five rays. In this system an impulse loses in effectiveness as it passes from the point of origin.

4. When *Antedon* is made to rest *oral* face down on the floor of an aquarium, oral flexion of all the rays, swimming movements, and righting result. *Antedon* is therefore negatively stereotropic with reference to its ventral side.

5. Excitation of the dorsal cirri results in *aboral* bending of all the rays. Stimulation of the cirri inhibits ventral flexion to the extent of preventing righting movements while on the other hand stimulation of the ventral surface inhibits the grasp reflex of the cirri. Thus oral and aboral sides of *Antedon* exhibit dynamic symmetry although structurally dissimilar.

I take this occasion to thank Professor Bottazzi of the physiological section of the Station for giving me free use of the laboratory. I also wish to express my appreciation of the excellent service rendered by Mr. Santarelli and his staff of the collecting department in procuring material for the work.

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4. Moore, A. R., *J. Gen. Physiol.*, 1918–19, i, 97; 1919–20, ii, 201.
5. See also Greenwood, M., *J. Physiol.*, 1890, xi, 594.
6. Moore, A. R., *Am. J. Physiol.*, 1910–11, xxvii, 207.
7. Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915–16, xxxix, 139.
8. Marshall, A. M., *Quart. J. Micr. Sc.*, 1884, xxiv, 507.
9. Jennings, H. S., Behavior of the lower organisms, New York, 1906, 273.
10. Note, however, Crozier, W. J., *Am. J. Physiol.*, 1914–15, xxxvi, 8.

HYDROGEN ION CONCENTRATIONS WITHIN THE ALIMENTARY TRACT OF INSECTS.

By W. J. CROZIER.

(From the Zoological Laboratory, Rutgers College, New Brunswick.)

(Received for publication, November 12, 1923.)

There has been a good deal of discussion as to the digestive and absorptive functions respectively pertaining to the several divisions of the intestinal tract of insects. The actual acidities prevailing in the various regions of the gut are of special moment. Among other points of interest, questions arise as to the conditions of enzyme activity within the gut, and as to the possible rôle of such conditions in connection with the absorption of drugs. Possible relations to the transformations of intestinal parasites (*e.g.* Herptemonads) are likewise suggestive and important.

Chiefly as a result of qualitative experiments involving ingestion of foodstuffs containing indicators such as litmus powder, it has been stated that the anterior portion of the intestinal lumen is in general alkaline in reaction, the midgut acid, the hinder portion of the intestine faintly acid or neutral in reaction. Much variation is evident, however, in the reports of different observers[1] using widely divergent sources of insect material. Thus Sanford,[2] repeating and extending observations and experiments of Petrunkewitsch, was able to show that the crop of the cockroach was alkaline to litmus, "neutral" to phenolphthalein (except after ingestion of olive oil, when it was sometimes found acid to litmus), while the stomach, corresponding to the condition reported by a number of observers, was acid. On the other hand, Jameson and Atkins[3] find in the

[1] A general summary of older work, with citations, is given by Jordan, H., Vergleichende Physiologie wirbelloser Tiere. I. Die Ernahrung, Jena, 1913.

[2] Sanford, E. W., Experiments on the physiology of digestion in the *Blattidæ*, *J. Exp. Zool.*, 1918, xxv, 355.

[3] Jameson, A. P., and Atkins, W. R. G., On the physiology of the silkworm, *Biochem. J.*, 1921, xv, 209.

silkworm that the contents of the fore part of the midgut are highly alkaline (pH = 9.0 to 9.8, full fed or fasting), the contents of the hindgut less alkaline (pH = 8.4); in the mature moth the midgut was distinctly acid (pH of contents = 5.2 to 5.8), perhaps due to waste products excreted into the intestine, since the imago does not feed. The reaction of the intestinal contents is difficult to interpret in the case of some insects, since the fluid food juices imbibed may largely determine the result; in *Flata*, for example, the mesenteric reservoir is acid to litmus, but so are the food juices, while in *Siphanta* the whole gut is said to be slightly alkaline to the same indicator.[4] Some further confusion in comparing different accounts seems to be due, in addition, to the lack of uniformity in naming parts of the insect digestive tract.

Particularly instructive observations can be made with certain translucent aquatic larvæ, such as young *Chironomus* larvæ and the early larval instars of *Psychoda* (diptera). These may be maintained for days in solutions of non-toxic sulphonephthalein indicators.

The indicators quickly permeate the alimentary tract. The larvæ may be removed to clear water for observation under the microscope, and the condition of the indicator determined in the living larva by inspection of the juice in the anterior and posterior portions of the gut, and of the fluid surrounding the peritrophic membrane which encloses the food in the mesenteron. By dissection with needles the intestine is readily extracted and examined separately; the peritrophic membrane with its contents may be removed and opened, and the malphigian tubules isolated and dissected in a drop of indicator. The effect of feeding, the result of varying the external alkalinity over a wide range, the action of agents affecting salivary discharge, and similar points may be in this way studied directly.

Larvæ of *Psychoda alternata* were chiefly used. The hemoglobin in *Chironomus* larvæ, seen greenish in hue when viewed in thin layer, may interfere with the judgement of indicator tints, and the *Psychoda* larvæ are of superior transparency. These two kinds of larvæ, however, gave results essentially identical. A "two color" indicator is

[4] Kershaw, J. C., The alimentary canal of *Flata* and other *Homoptera*, *Psyche*, 1913, xx, 175.

particularly serviceable. Brom-thymol blue was most convenient.[5] Its non-toxicity is evidenced by the ecdysis and pupation of larvæ growing in concentrated solutions of the dye, and their final emergence as imagines. Observations made with brom-thymol blue were checked quantitatively by others independently made with phenol red, neutral red, and brom-phenol blue; possible errors due to the color of food juices were thus eliminated.

In the actively feeding larva the most anterior region of the alimentary tract contains fluid of pH 7.0 to 7.2. The secretion of the salivary glands is on the whole faintly alkaline, rather than neutral. The lumen of the cardiac chamber of the stomach shows tints connoting pH 6.2 to 6.7, in different cases. The variation in pH I take to be possibly indicative of varying degrees of secretory activity. There is a sharp line of demarcation at the posterior border of the cardiac chamber between this zone of pH 6.2 to 6.7 and the immediately caudad mesenteron ("stomach" of text-books); in the mesenteron the pH usually observed, in the fluid bathing the peritrophic membrane, is 7.5 (range, 7.2 to 7.8). The sharpness of this division is made vividly obvious by the yellow and blue colors of brom-thymol blue on either side of the pyloric sphincter. With *Chironomus* especially it frequently happens, also, that food is regurgitated from mesenteron into cardiac chamber; in such circumstances the blue material from the mesenteron is instantly turned yellow. When food from the cardiac chamber is ejected from the mouth into a medium of pH about 7.4 containing brom-thymol blue, it is seen yellow, and the diffusion of acid from it can be watched.

The hindgut ("intestine") is acid in reaction, the pH observed varying from 6.0 or less to 6.6. But this, as will be pointed out, is true only of young larvæ.

The observations are summarized in Fig. 1.

[5] The numerical pH values obtained with this indicator may be subject to "salt correction" of unknown but probably quite small magnitude. The tints observed in the larvæ, or in the freshly isolated intestine, were compared with those in test-tube standards. A convenient method of doing this, in the case of minute objects under the microscope, has been described by Pantin, C. F. A., *Nature*, 1923, iii, 81.

The magnitudes of the pH values, and the succession of alterations in these values down the course of the gut, do not differ significantly among larvæ maintained in solutions of pH 6.0, 6.4, 6.8, 7.4, 7.6, or 8.0. Therefore they represent the normal, independent condition of the intestine. It will be noticed that in these larvæ the ingested food is subjected first to a faintly alkaline salivary juice, passes then through a distinctly acid cardiac chamber, thence to a no less distinctly alkaline portion of the gut. This alkaline region, the mesenteron, often termed "stomach" (ventriculus) is the portion of the gut through which food passes most slowly, and seems in fact to be the region of most active absorption. The hindgut (proctodeum), often labelled "intestine," appears of greatest importance as an excretory channel. From this standpoint the course of alimentation in these larvæ is not dissimilar to that in a vertebrate.

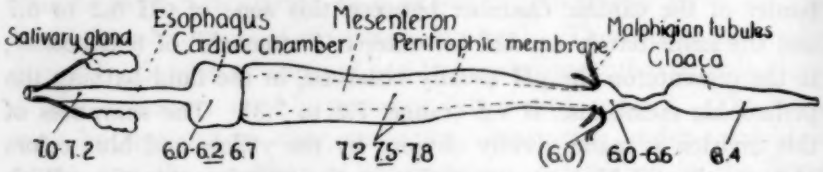

FIG. 1. Diagram of intestinal tract of dipterous larvæ (*Psychoda*, *Chironomus*) showing pH values observed in the several divisions. Where a range of acidities is indicated, the commonest value is underlined. The pH of the malphigian tubules is as a rule less than 6.0.

That the mesenteron is a major locus of absorption is shown by several additional facts. The peritrophic membrane is freely permeable to the indicators used, as indeed Eidmann's[6] results would have suggested. The contents of the peritrophic tube, when the isolated tube is dissected out, appear to have the same alkalinity as that noted in the fluid which normally encloses it. The cells composing the wall of the mesenteron are the only cells in the gut which appear to absorb the dye. The stained cells show a color indicating[7] (brom-thymol blue) pH 6.4. In *Chironomus* larvæ the

[6] Eidmann, H., *Biol. Zentr.*, 1922, xlii, 429.

[7] Shackell (Shackell, L. F., *J. Biol. Chem.*, 1923, lvi, 891, foot-note 2) implies that the combination of *brom-phenol blue* with proteins of egg white does not interfere with its correct color changes according to pH.

fluid surrounding the peritrophic membrane is often confined to a narrow annulus at the anterior end of the mesenteron, from which level it is rapidly run over the peritrophic membrane posteriorly and then anteriorly again by a kind of peristalsis.

The hindgut of the larvæ studied is probably of minor significance for digestion and absorption. Its acidity (pH 6.0 to 6.6) is due to the discharge from the malphigian tubules. The contents of the tubules are quite markedly acid. Older larvæ, preparing for pupation, show the tubules plugged proximally by urate deposits. In such larvæ the lumen of the hindgut is less acid, showing pH 6.8 to 7.1.

SUMMARY.

Larvæ of *Psychoda* and of *Chironomus* (diptera) maintained in solutions of appropriate indicators show that the typical acidities (pH) prevailing within the several regions of the digestive tract are: esophagus 7.1; cardiac chamber 6.2; mesenteron 7.5; the latter being functionally an intestine. The acidity of the hindgut, pH 6.4, is due to the discharge of the malphigian tubules.

THE EFFECT OF LABORATORY AGE UPON THE PHOTOTROPIC REACTIONS OF LIMULUS.

By WILLIAM H. COLE.

(*From the Laboratory of Biology, Lake Forest College, Lake Forest, Illinois.*)

(Received for publication, November 19, 1923.)

In a previous paper[1] it was shown that *Limuli* "freshly collected" are predominantly positive to photic stimulation; that they show positive circus movements when a single eye is stimulated; and that these reactions are easily accounted for by the tropism hypothesis. It was also stated that considerable irregularity, and sometimes reversal, in the phototropic reactions may occur, especially in "hungry" animals, and in those stimulated severely by other means than light. More recently Northrop and Loeb[2] reported the results of their experiments on the phototropism of *Limulus*, in which only negative reactions were discussed. This apparent discrepancy regarding the sign of the phototropic reaction of *Limulus* called for explanation, and the experiments[3] reported here were done. It is stated[4] that only ten out of forty-eight specimens could be used, and it is suggested that lack of response to photic stimulation might have been caused by unfavorable laboratory conditions. In the experiments at least 1 week had elapsed between the time of collecting the animals and the time of making the tests. These facts lead to the belief that changes in the phototropism of *Limulus* are closely linked with, and probably dependent upon, the laboratory age of the specimens. Accordingly tests were made daily upon recently collected individuals, and upon the same ones at each 24 hour interval thereafter over a period of 1 week. The first tests upon each lot of animals were made within 6 hours after the speci-

[1] Cole, W. H., *J. Gen. Physiol.*, 1922–23, v, 417.

[2] Northrop, J. H., and Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 581.

[3] The tests were made in 1923 at the Marine Biological Laboratory, Woods Hole, Massachusetts and the writer wishes to express his thanks to the Director for his accommodations.

[4] Northrop and Loeb,[2] p. 583.

mens were collected from their natural habitat. All of them were small, varying from 20 to 60 mm. in width, the majority being about 30 mm. wide. Between the daily tests all the animals were kept in large aquaria with running sea water, the temperature and pH of which varied but slightly from that of the water in which the *Limuli* were found.

In various positions they were subjected to the directive stimulation of a beam of light, 30 mm. in diameter, coming from a 25 watt Mazda

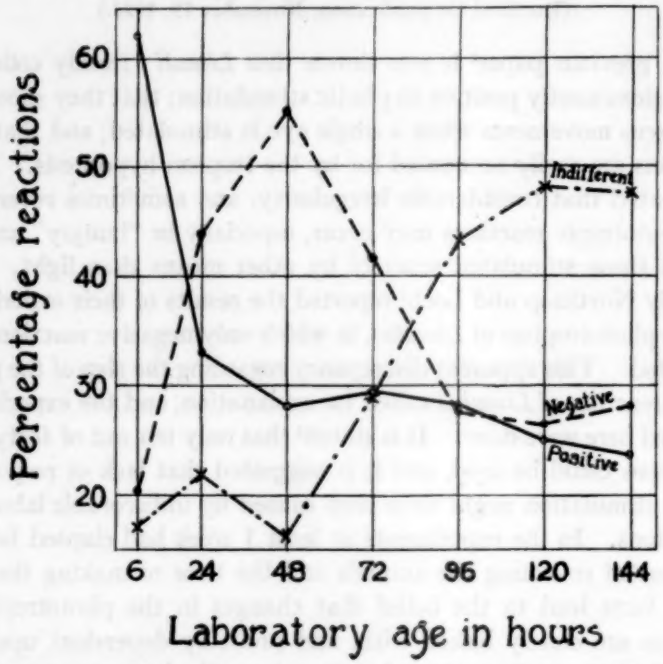

FIG. 1. Graphs showing the percentages of positive, negative, and indifferent reactions of *Limuli* at successive 24 hour intervals of laboratory confinement.

lamp at a distance of 14 cm. from the center of the shallow, glass crystallizing dish. Each animal was tested separately three successive times. If there was any doubt as to the sign of the response, the animal was set aside and tested again later three more times. If doubt still existed, the animal was classified as indifferent. The number of individuals in each lot showing positive reactions, and the number showing negative reactions were recorded. The remainder

were recorded as indifferent. The percentages for each kind of reaction for each lot were calculated. Fresh animals exhibited an accuracy and a precision of orientation which were never seen in animals of a laboratory age of 24 hours or more.

Fig. 1 shows the graphs in which the percentages of positive, negative, and indifferent reactions are plotted against laboratory age. It is seen that during the first 24 hours positive reactions markedly decrease, from about 62 to about 33 per cent. During the next 24 hours there is a slight decrease to 28 per cent, a figure which changes but little during the rest of the week. It is during the first 24 hours, therefore, that laboratory confinement profoundly affects the animal's positive reactions to light. Negative reactions also show variation, but in a different way. They increase from 20 to 55 per cent during the first 48 hours, and then show a steady decrease to about 28 per cent during the second 48 hours. Thereafter the percentage remains practically constant. In the case of indifferent reactions there is fluctuation during the first 48 hours, followed by a steady increase to approximately 50 per cent at the end of 120 hours. At the close of a week's laboratory confinement, therefore, it may be predicted that about 50 per cent of a *Limulus* population will be indifferent to light; about 30 per cent will be negative and about 20 per cent positive, although when first brought into the laboratory they showed 60 per cent positive, 20 per cent negative, and 20 per cent indifferent reactions. From the graphs in Fig. 1 it may be determined how long *Limulus* should be kept in the laboratory to exhibit the maximum number of reactions of the kind desired.

The determination of the factors of the laboratory environment which might be responsible for the changes in reactions was not attempted, the purpose having been merely to demonstrate that the changes do occur, and to clear up the discrepancy referred to above.

THE EFFECT OF PHOSPHATES ON RESPIRATION.

By CHARLES J. LYON.

(From the Department of Biology, Dartmouth College, Hanover, and from the Marine Biological Laboratory, Woods Hole.)

(Received for publication, November 21, 1923.)

This is a preliminary report of a study of the action of phosphate upon the production of CO_2 by plants. The phosphate caused a marked and sustained acceleration of both the aerobic and anaerobic respiration.

The experiments were carried on by the use of the apparatus described by Osterhout[1] modified by replacing the rubber valves by two glass bead valves that handle a large current of air more easily and with less danger of a reverse current that often ruins an experiment. The third tube suggested by Osterhout[2] to hold the reagent with a buffer effect while the normal is being determined, was placed so that all CO_2 passed through it on the way to the indicator tube. An extra section of rubber tubing extending between the glass tubes (with its ends flush with the lower side of the rubber stoppers) is clamped off until the normal has been determined. Without opening the system or stopping the air current, the clamp is removed and the tube of phosphates is tilted until the solution passes over into the reaction chamber.

The well known function of phosphate in alcoholic fermentation as shown by Harden and Young (1910) and the generally accepted theories as to the relations between fermentation and respiration make it advisable to use modern methods in studying respiration as it may be affected by phosphates, with the pH and other factors carefully controlled. Palladin[3] believed that respiration is accelerated by phosphate but most of his authority for this seems to lie in the

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1919–20, ii, 1.

[3] Palladin, V. I., Plant physiology, Philadelphia, 1918, 194.

writings of a number of contemporary workers, L. Iwanoff,[4] N. Iwanoff,[5] Zaleski,[6] and others. Study and comparison of these papers only bring out the conflicting conclusions, the lack of data as to salts and concentrations employed and the pH of the reaction mixtures, and the inaccuracy and incompleteness of such experiments due to the failure to follow the time curves as can be done with the apparatus used in my experiments.

More recently there has appeared a paper by Witzemann[7] in which phosphate is shown to be a catalyst for the oxidation of glucose by H_2O_2. This would lead one to suspect that phosphate might accelerate aerobic respiration. My experiments (which were well started before this paper was published) show this to be true. In fact, certain of my experiments to be reported later seem to indicate that phosphate may catalyze an oxidation in the absence of a peroxide.

Recent papers by Embden and his students[8] and by Meyerhof[9] have appeared as the result of studies of the part played by H_3PO_4 in the production of the energy used in the muscles of animals. A compound containing carbohydrate and phosphoric acid is considered to be the controlling substance of muscular contraction. To account for these results, Meyerhof in particular has outlined a scheme of oxidation of glucose that involves the formation and decomposition of a hexosephosphate in both the aerobic and the anaerobic phases. In so far as it is possible to compare the effects of phosphate action in my experiments on plant respiration with those of H_3PO_4 on muscular action, the action appears to be the same in both cases, an acceleration of the metabolic processes involved. But it is not clearly indicated that the series of chemical reactions in plant respiration are the same as those suggested to explain the rôle of H_3PO_4 in oxidation in animal

[4] Iwanoff, L., *Biochem. Z.*, 1910, xxv, 171.

[5] Iwanoff, N., *Biochem. Z.*, 1911, xxxii, 74.

[6] Zaleski, W., *Ber. bot. Ges.*, 1910, xxviii, 319. Zaleski, W., and Reinhard, A., *Biochem. Z.*, 1910, xxvii, 450.

[7] Witzemann, E. J., *J. Biol. Chem.*, 1920–21, xlv, 1.

[8] Embden, G., and others, *Z. physiol. Chem.*, 1921, cxiii. Embden, G., and Lawaczeck, H., *Biochem. Z.*, 1922, cxxvii, 181; and other papers.

[9] Meyerhof, O., *Arch. ges. Physiol.*, 1920, clxxxv, 11; 1921, clxxxviii, 114; 1921, cxci, 128; and other papers.

muscles, though they have this in common; both the aerobic and the anaerobic phases are affected.

The first experiments were carried out with *Elodea canadensis*. Fig. 1 shows the results of three typical experiments in which different concentrations of neutral phosphate solutions[10] were used. For convenience these solutions will be referred to as having a definite concentration in the sense that when a 0.1 M solution is mentioned a solution is meant which was obtained by mixing 0.1 M monosodium phosphate with 0.1 M disodium phosphate.

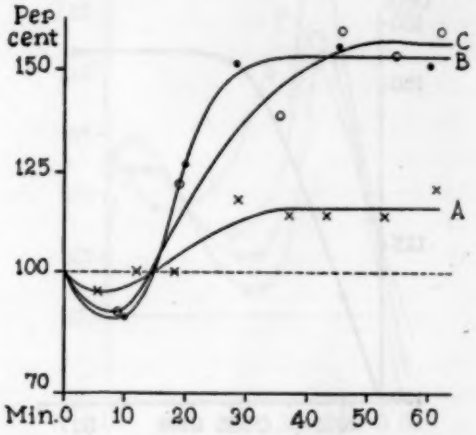

FIG. 1. The effect of neutral phosphate solutions on the production of CO_2 by *Elodea*. Each curve represents a typical experiment. Curve *A* shows the effect of a concentration of 0.021 M; Curve *B*, 0.085 M; Curve *C*, 0.106 M. The normal rate before the addition of phosphate is taken as 100 per cent.

An experiment with potassium salts gave identical results with those obtained with the sodium salts which were used throughout the work. The first readings usually fall below normal: this is either the result of a preliminary depressing action on respiration or is due to

[10] From a paper by Beysel, W., and Löb, W., *Biochem. Z.*, 1915, lxviii, 368. I obtained Sörensen's table for the mixtures of equimolecular solutions of monosodium and disodium phosphates to give solutions of definite pH. Interpolation and checks with indicators (phenolsulphonephthalein and rosolic acid) showed that a mixture of 6.2 parts of Na_2HPO_4 plus 3.8 parts of NaH_2PO_4 gives a neutral solution.

the buffer effect of the phosphate mixture being slightly changed by the dilution of the mixture as the result of its transfer to the reaction chamber. However, the equilibrium is soon reestablished and the curve rises, flattening out after an hour. Fig. 2 shows the concentration curve of acceleration after an hour's exposure, each point being the average of several experiments. Higher concentrations gave plasmolysis and were not tested. At the point where the curve becomes horizontal the acceleration is 56 per cent.

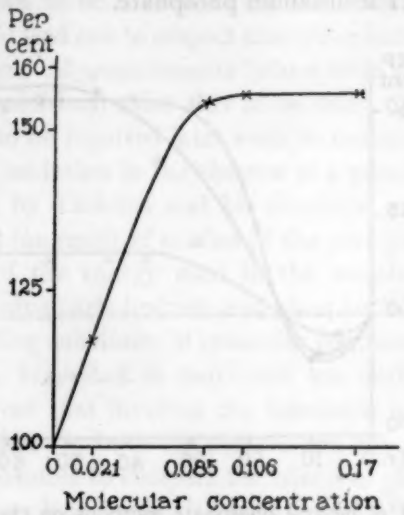

FIG. 2. The rate of production of CO_2 by *Elodea* in different concentrations of neutral phosphate solutions after an exposure of 1 hour. Each point is obtained by averaging from four to nine experiments (such as are shown in Fig. 1). Probable error of the mean is less than 3.5 per cent of the mean. The normal rate before the addition of phosphate is taken as 100 per cent.

After it was shown that all concentrations of neutral phosphate solutions as high as 0.17 M markedly accelerate the rate of production of CO_2, it became of interest to see how long this effect lasted. Many experiments were continued for 2 and 3 hours and in general the rate remained the same. In order to test it for longer periods the plants were removed from the reaction chamber after a measurement had been taken, and kept in an open dish of neutral phosphate mixture until about 2 hours before another measurement was desired. Then

the CO_2-free current was run through them in the closed system for 1½ to 2 hours before the next observation was made. This removed all accumulation of CO_2, even when the plants had been left for 10 hours, as was shown by the constancy of the readings obtained.

Fig. 3 shows the individual curves and the average of three experiments, all of which agreed in general but varied in the time required

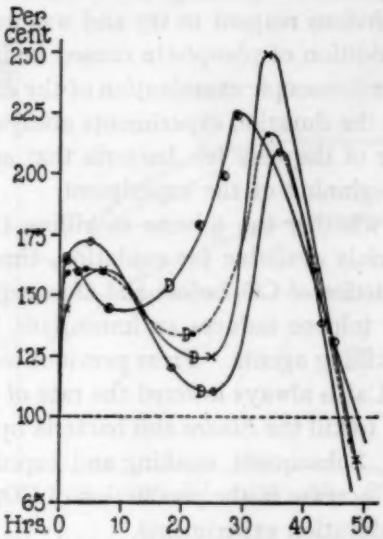

FIG. 3. The individual time curves and the average curve (dotted line) of three typical experiments on the effect of a 0.106 M neutral phosphate solution on the production of CO_2 by *Elodea*. The *D* on each curve indicates the approximate time of death of the cells as tested by plasmolysis. Probable error of the mean is less than 10 per cent of the mean. The normal rate before the addition of phosphate is taken as 100 per cent.

to arrive at corresponding parts of the curve. The preliminary acceleration gradually lessens, then rises sharply to a new maximum, and later is rapidly lost. Plasmolytic tests for the death point showed that death occurred 18 to 24 hours after exposure to the phosphate, at the time when the curve had nearly or just reached its first minimum. Thus the second rise followed the death of the plant cells[11] and oxida-

[11] Regarding respiration after death see Haas. A. R. C., *Bot. Gaz.*, 1919, lxvii, 347.

tion appeared to go on under the influence of the phosphate. The rate of production of CO_2 fell below the normal after several hours; presumably all the easily oxidisable substances were used up.

To meet the possible objection that rapid multiplication of bacteria after the death of the *Elodea* was responsible for the second rise, efforts were made to kill the *Elodea* in such a way as to kill the bacteria and the *Elodea* and leave the enzymes of the latter unharmed. Toluene is, of course, the obvious reagent to try and was used with the result that subsequent addition of phosphate caused no increase in production of CO_2. Yet microscopic examination of the *Elodea* leaves before and after death in the duration experiments always failed to show an increase in number of the very few bacteria that are not removed by washing, at the beginning of the experiment.

In order to see whether the toluene in killing the plants had not used up the materials available for oxidation, time curves were obtained of the production of CO_2 before and after exposure to toluene.[12] It was found that toluene induces an immediate, large acceleration, as do many other killing agents. From previous work[13] it was known that a solution of $CaCl_2$ always lowered the rate of production of CO_2 so it was here used to kill the *Elodea* and bacteria by a 2 hour exposure to a 1 M solution. Subsequent washing and exposure to phosphate resulted in a large increase in the production of CO_2. This confirmed the results of the duration experiments.

The experiments described above dealt with normal respiration which may be regarded as the sum of the anaerobic and aerobic processes going on in the plant. When the apparatus was filled with hydrogen gas, no production of CO_2 was observed in several experiments on *Elodea*. This shows that phosphate accelerates the aerobic phase. In order to test the effect on the anaerobic phase it was necessary to use another plant and sterile wheat seedlings (caulicle $\frac{1}{4}$ to $\frac{3}{4}$ inch long) were selected. Fig. 4 shows the average curves for the normal (or combined) respiration and for the anaerobic phase alone, as affected by phosphate. It is evident that much of the production of CO_2 is to be assigned to the anaerobic phase, as is shown

[12] Toluene destroys rubber valves and only the bead valves made these experiments possible. They will be reported later.

[13] Lyon, C. J., *Am. J. Bot.*, 1921, viii, 458.

by the fact that the anaerobic is only 28 per cent less than the aerobic (when no phosphate is added). The exposure to the phosphate solution results in an increase in production of CO_2 whether the oxygen is present or absent.

Curve *A* is for the normal respiration while Curves *B* and *C* are for the anaerobic phase alone. Curve *C* is Curve *B* moved up by 28 per cent so that it starts at 100 per cent and is more easily compared

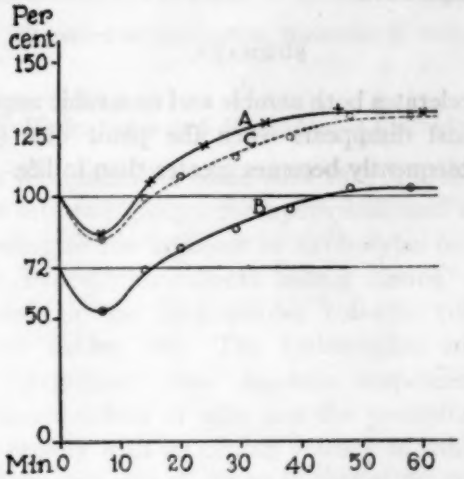

FIG. 4. The effect of a 0.04 M neutral phosphate solution on the production of CO_2 by wheat seedlings in air (Curve *A*) and in hydrogen (Curves *B* and *C*). The normal rate is taken as 100 per cent for Curve *A*. For Curve *B*, the average rate before exposure to the phosphate was 72 per cent of the rate before the oxygen was removed; hence the curve for anaerobic action starts at 72 per cent. Curve *C* is Curve *B* with its first point moved up to 100 per cent for comparison with Curve *A*. Average of three experiments for Curve *A* and of four for Curve *B;* probable error of the mean is less than 3.5 per cent (except for one point which is less than 9 per cent) of the mean.

with Curve *A*. It will be seen that the levels of acceleration are about the same at the end of the hour, the exact figures being 136 per cent for the normal (combined) and 132.25 per cent for the anaerobic alone. This proves the acceleration of the anaerobic phase of wheat seedlings and shows again (but less conclusively) that the aerobic phase is accelerated for if only the anaerobic were affected the

per cent of acceleration of the combined respiration would have been less than that for the anaerobic alone and not slightly more as the curves show.

The nature of the phosphate action will be further studied by experiments now being carried out. A full report and discussion of the above results is reserved until all the information is obtained. It seems probable, however, that phosphates will be shown to be necessary for plant respiration.

SUMMARY.

Phosphate accelerates both aerobic and anaerobic respiration. The acceleration almost disappears when the plant dies (in phosphate solution) but subsequently becomes greater than in life.

HYDROPHILIC AND HYDROPHOBIC COLLOIDS AND THE INFLUENCE OF ELECTROLYTES ON MEMBRANE POTENTIALS AND CATAPHORETIC POTENTIALS.

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I.

Hydrophilic and Hydrophobic Colloids.

It has been recognized for some time that it is necessary to subdivide colloids into two groups, the hydrophilic and the hydrophobic colloids, according to the influence of electrolytes on their precipitation. To the hydrophilic colloids belong among others proteins and soaps, and to the hydrophobic colloids, collodion, mastic, graphite, metal oxides, etc. The hydrophobic colloids (such as mastic) are precipitated from aqueous suspensions by comparatively low concentrations of salts and the precipitating effect of a salt increases rapidly with increasing valency of that ion of the salt which has the opposite sign of charge to that of the particle. Hydrophilic colloids, such as genuine crystalline egg albumin or gelatin, are only precipitated in very high concentrations of electrolytes and the sign of charge of the precipitating ion has no relation to the sign of charge of the protein. All attempts to account for this difference in the action of electrolytes on the two kinds of colloids by a common theory have failed for the reason that the forces which prevent the particles from coalescing in an aqueous solution are entirely different in the two cases. Hydrophilic colloids, such as genuine proteins, form true solutions in water, *i.e.* the forces by which the protein molecules or protein ions are kept in solution are the strong forces of chemical affinity between molecules of water and protein;[1] while the forces

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 243. Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921–22, iv, 187. Loeb, J., *Arch. néerl. physiol.*, 1922, vii, 510. Cohn, E. J., and Hendry, J. L., *J. Gen. Physiol.*, 1922–23, v, 521.

which prevent particles of hydrophobic colloids from coalescing are the weak forces of electrostatic repulsion due to the existence of an electrical double layer surrounding each particle. This explains why the influence of electrolytes on the precipitation of the two types of particles is so different that it cannot be covered by the same theory.

It has been known for some time that certain hydrophilic colloids prevent the precipitation of suspensions of hydrophobic colloids by low concentrations of salts and this effect has been called the protective action of colloids. Zsigmondy has especially investigated the protective action of gelatin on suspensions of gold in water, and he and others reached the conclusion that this is due to the formation of a film of gelatin around the gold particle.[2] The writer has shown that this protective action of a film of gelatin on the surface of hydrophobic colloids is due to a change in the nature of the forces which prevent the particles from coalescing when they collide. While particles of gold or collodion are prevented from coalescing only when the P.D. of the electrical double layer surrounding the particles exceeds the critical value of about 12 to 15 millivolts, the same particles when coated with gelatin will not coalesce even when their cataphoretic P.D. is zero, for the reason that the particles coated with gelatin are kept from coalescing not by the electrostatic repulsion due to their electrical double layer but by the forces of attraction between the polar (or "aqueous") groups of the molecules of gelatin and water. The writer was able to prove this by a comparison of the effect of electrolytes on the precipitation of solutions of gelatin and of suspensions of particles of collodion coated with gelatin.[3] It was found that the effect of electrolytes is qualitatively and quantitatively the same in both cases. Since gelatin forms true solutions in water (like the amino-acids from which it is built up), it follows that the protective action of gelatin is due to the attraction between gelatin and water. It follows further that it would be wrong to assume that all suspensions of solid particles in water are hydrophobic colloids; solid particles of gelatin in water or gelatin-coated particles of collodion, gold, etc., are hydrophilic suspensions, since the forces which keep

[2] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd edition, 1918.

[3] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479.

the particles from coalescing are the strong forces of attraction between water and gelatin which are destroyed only by high concentrations of salt. The sign of charge of the precipitating ion bears in this case no relation to the sign of charge of the particle.[4]

Crystalline egg albumin forms also true solutions in water and hence is a hydrophilic colloid, but loses its high affinity for water and becomes practically insoluble when boiled. The particles of such boiled or "denatured" egg albumin are prevented from coalescing only by the electrostatic forces of repulsion between the particles due to their double layers, and they are therefore hydrophobic colloids. The writer has shown that when collodion particles are coated with genuine crystalline egg albumin, the albumin becomes denatured in the film and the particles are kept from coalescing only by the weak forces of electrostatic repulsion due to the electrical double layers. Crystalline egg albumin has little or no protective action on hydrophobic colloids. The fact that crystalline egg albumin becomes insoluble when it forms a film on the surface of collodion particles may be due to a change in the configuration or orientation of the albumin molecules in the film.[3]

While it is true that the influence of salts on the precipitation of the two types of colloids cannot be explained by the same theory, it does not follow that there exists no analogy in the effects of salts on any of the colloidal properties of the two groups. Such an analogy actually exists if instead of the action of electrolytes on precipitation the action of electrolytes on certain other properties of the two types of colloids is compared.

It has been shown that genuine proteins are true crystalloids in regard to chemical reactions, solubility, and probably kindred proper-

[4] Some authors use the term emulsoids for hydrophilic colloids, and suspensoids for hydrophobic colloids. The terms emulsoids and suspensoids should be abandoned in this case, since proteins form true solutions in water which have nothing in common with emulsions. Furthermore, micelles of gelatin or particles of collodion coated with gelatin are kept in solution by the same forces which are responsible for true solution and it would be wrong to apply to them the term suspensoids. The terms hydrophilic and hydrophobic colloids are logical and lead to no contradiction.

ties such as surface tension. Genuine proteins show colloidal behavior only when their large ions are prevented from diffusing through membranes or gels which are readily permeable to the small ions of ordinary electrolytes. When, under such conditions, osmotic equilibrium is established between a protein solution or a protein gel and the outside solution, the total molar concentration of the diffusible ions is greater inside the protein solution or the protein gel than in the surrounding aqueous solution free from protein, and this excess of the total molar concentration of diffusible ions inside the protein solution or gel over that outside varies with the concentration and nature of an electrolyte. This and nothing else is the cause of the influence of electrolytes on the colloidal behavior of proteins and this was proved by the fact that when this difference in the molar concentration of the diffusible ions inside and outside the protein solution or gel is known, the colloidal properties can be predicted quantitatively. The colloidal properties of proteins thus affected are membrane potentials, osmotic pressure, swelling, and that type of viscosity which depends on the swelling of protein micelles.[5]

It is found that these four properties of proteins are influenced in a similar way by electrolytes as is the stability of hydrophobic colloids. Low concentrations of electrolytes suffice to bring the values of the membrane potentials, the osmotic pressure, the colloidal type of viscosity of protein solutions, and the acid or alkali swelling of protein gels to zero, and the active ion of an electrolyte has always a sign of charge opposite to that of the protein ion.

It is natural then to raise the question whether this analogy in the effects of electrolytes on the colloidal behavior of hydrophilic and hydrophobic colloids is merely accidental or whether it can be explained by a common theory.

In order to simplify our problems we will compare the influence of electrolytes on the membrane potentials of hydrophilic colloids (proteins) and on the cataphoretic potentials of hydrophobic colloids (particles of collodion and mastic).

In order to avoid confusion, it will be necessary to distinguish between the charging effect and the depressing effects of an electrolyte

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

on the two types of potentials. The charging effect of electrolytes is different for the two kinds of potentials while the depressing effect is similar.

The charging effect of electrolytes on the membrane potentials of proteins is confined to acids and alkalies. This is due to the fact that the charging effect depends in this case on the formation of protein ions, which are responsible for the Donnan equilibrium on which the colloidal behavior of proteins rests. The ionization of a protein is a minimum at the isoelectric point and the addition of acids and alkalies to isoelectric protein causes a formation of protein salt dissociating electrolytically. The addition of a neutral salt of the pH of the isoelectric point of protein to an isoelectric solution of protein, however, causes no formation of protein salt and protein ions. For this reason neutral salts have no charging effect on the membrane potentials of protein solutions.[5]

The addition of little acid or little alkali to a protein solution causes at first an increase in the ionization and membrane potentials of the protein solution until a maximum potential is reached. When this maximum is once reached, a further addition of acid or alkali will cause a depression of the membrane potentials of protein solutions.

The charging effect of electrolytes on the *cataphoretic* P.D. is not confined to alkalies and acids, but is produced also by salts at neutral reaction.[6] The reason for this is that the cataphoretic P.D. of particles of non-electrolytes like collodion or mastic is not caused by a salt formation and ionization of this material but by the formation of an electrical double layer between particle and water, one film of which adheres to and moves with the particles. This film has as a rule a negative charge in the case of particles of collodion and mastic owing to the fact that as a rule negative ions are driven in excess into the film directly enveloping the particle; while an equal excess of cations exists in the adjacent film. The forces which cause this unequa distribution of oppositely charged ions between the two films of the double layer may be either forces inherent entirely in the water or forces of attraction between particles and one of the two oppositely charged ions; or a combination of both forces. For this reason salts

[6] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109, 479, 505; 1923–24, vi, 105, 215.

as well as alkalies or acids can act as charging electrolytes for the cataphoretic P.D. of particles of dielectrics in water.[6]

The cataphoretic P.D. seems never to exceed a maximal value of about 70 millivolts, but the maximal P.D. may be considerably less than 70 millivolts, according to the nature of the particle and the nature of the charging electrolyte. If the maximal P.D. characteristic for the particle and the charging electrolyte has been reached, the addition of any electrolyte, salt, acid, or alkali will have a depressing effect.[7]

There can therefore be no common theory for the charging effects of electrolytes on membrane potentials of protein solutions and on the cataphoretic P.D. of particles of dielectrics. The only question which can be raised is whether the depressing effect of electrolytes on the two potentials can be derived from a common theory. In order to answer this question it will be necessary to compare the depressing effects of salts on the two types of potentials.

II.

The Valency Rule for the Depressing Effect of Salts on Membrane Potentials.

In these experiments the charging electrolyte was NaOH. 2 gm. of originally isoelectric gelatin were dissolved in 100 cc. of water containing 16 cc. of 0.1 N NaOH which brought the pH of the solution to exactly 11.0. These were diluted with equal parts of solutions of five different salts, LiCl, NaCl, KCl, $CaCl_2$, and $BaCl_2$, also of pH 11.0 but of different molar concentration. Collodion bags were filled with 50 cc. of this mixture and submerged in an aqueous solution free from protein but containing the same salt in the same molar concentration as that in the protein solution; and, moreover, this outside solution was also brought to pH 11.0 through the addition of NaOH. To prevent the effect of the CO_2 of the air on the pH, the outside solution was kept in closed flasks in which the air communicated with the outside air through tubes filled with soda lime. After about 5 hours, osmotic equilibrium was established and the membrane potentials between the inside gelatin solution and the outside aqueous solution were measured with the Compton electrometer as described.

[7] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 215.

Fig. 1 gives the effect of the five salts on the membrane potentials. The abscissæ are the concentrations of the salts, the ordinates, the P.D. in millivolts. It is obvious that the valency rule holds as had already been shown in previous publications on the subject. The values for the depressing effect of LiCl, NaCl, and KCl on the membrane potentials of Na gelatinate solutions lie all on one curve, and so

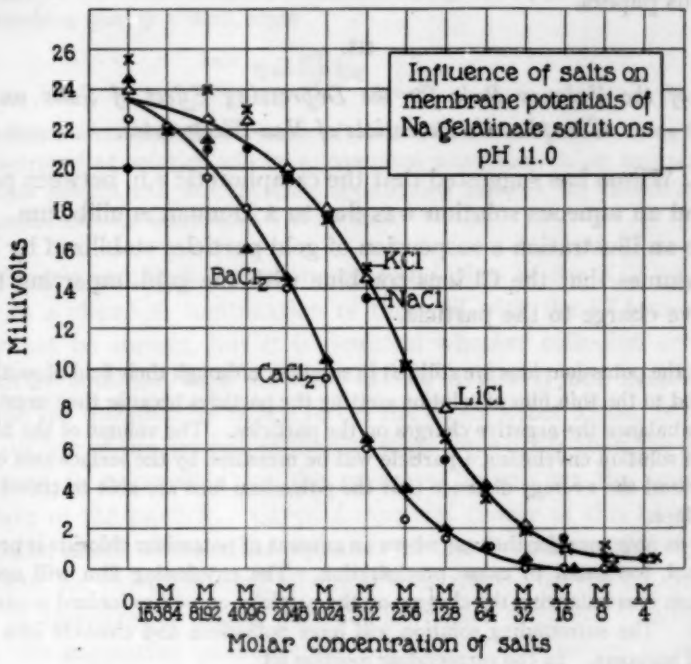

FIG. 1. Depressing effect of salts on the membrane potentials of a 1 per cent solution of Na gelatinate at pH 11.0. The abscissæ are the molar concentrations of salts, ordinates, the membrane potentials in millivolts. Only the valency but not the chemical nature of the cation determines the depressing effect.

do the values for $CaCl_2$ and $BaCl_2$.[8] In this case the protein solution is negatively charged and hence the depressing effect increases with the increasing valency of the cation of the depressing salt. Only the valency of the cation of a salt but not the chemical nature of the cation influences the membrane potentials between solutions of metal gela-

[8] This figure is taken from Mr. Kunitz's doctor's thesis.

tinates and an outside aqueous solution. Such a valency rule seems to be one of the proofs that the membrane potentials are due to a membrane equilibrium, since (as far as the writer is aware) only those properties which depend upon membrane equilibria show this exclusive influence of valency and complete absence of influence of the chemical nature of the ion. This has been discussed sufficiently in previous papers.

III.

Proof of the Valency Rule for the Depressing Effect of Salts on the Cataphoretic Potentials of Non-Electrolytes.

J. A. Wilson has suggested that the cataphoretic P.D. between particles and an aqueous solution was due to a Donnan equilibrium. He uses as an illustration a suspension of gold particles stabilized by KCl and assumes that the Cl ions combine with the gold imparting their negative charge to the particle.

"But the potassium ions are still left in solution, although their field of motion is restricted to the thin film of solution wetting the particles because they must continue to balance the negative charges on the particles. The volume of the film of aqueous solution enveloping a particle will be measured by the surface area of the particle and the average distance that the potassium ions are able to travel from the surface.

"Let us now consider the case where an amount of potassium chloride is present in the sol. too small to cause precipitation. The enveloping film will contain potassium ions balancing the charges on the particles as well as ionized potassium chloride. The surrounding solution will have potassium and chloride ions only in equal numbers. In the surrounding solution let

$$x = [K^+] = [Cl']$$

in the enveloping film let

$$y = [Cl']$$

and

$$z = [K^+] \text{ balanced by charges on the particles.}$$

whence

$$y + z \text{ represents the total concentration of potassium ion.}$$

As was shown in the discussion of Donnan's theory, the product $[K^+] \times [Cl']$ must have the same value both in the enveloping film and in the surrounding solution at equilibrium. Hence

$$x^2 = y\,(y + z).$$

The surface layer of solution will have a greater concentration of ions than the surrounding solution by the amount $2y + z - 2x$. This unequal distribution of ions will give rise to a difference of potential between the enveloping film and the surrounding solution whose measure is

$$E = \frac{RT}{F} \log \frac{x}{y} = \frac{RT}{F} \log \frac{2x}{-z + \sqrt{4x^2 + z^2}}$$

But now, if we increase x without limit while z remains constant, E must decrease, approaching zero as a limit, since

$$\lim_{x = \infty} E = \frac{RT}{F} \log \frac{2x}{\sqrt{4x^2}} = 0.$$

It is thus evident that the difference of potential between the enveloping film and the surrounding solution will be a maximum when there is no free potassium chloride present and will decrease, approaching zero, as the concentration of potassium chloride is increased without limit."[9]

Wilson's assumption that the charging effect of the KCl on gold is due to a chemical combination of the gold with the Cl ions may or may not be correct, but it is doubtful whether collodion or mastic particles combine with Cl ions. It is, however, not necessary to decide the question by which forces the Cl (or other anion) is held on the surface of the particle as long as some force other than the osmotic pressure is responsible for the excess of anions on the immediate surface of the particle. Given a maximal charge of this nature, the question arises whether the equation for the Donnan equilibrium can account for the depressing effects of electrolytes on the cataphoretic P.D. of collodion or mastic particles as Wilson suggests. In order to test this suggestion quantitatively, it would be necessary to measure three quantities, first, the cataphoretic P.D.; second, the value of z, *i.e.* the concentration of K ions bound by the Cl ions which are "adsorbed" by the solid particles; and third, the value of x, *i.e.* the molar concentration of K and Cl ions in the bulk of the solution. Only the first and the third of these three quantities can be measured directly, while it is impossible to measure z directly.

There is, however, an indirect method of testing Wilson's suggestion. If it is true that the depressing effect of electrolytes on the cataphoretic

[9] Wilson, J. A., The chemistry of leather manufacture, New York, 1923, 128–129.

P.D. finds its explanation in the Donnan equilibrium, we must expect that only the valency but not the chemical nature of the active ion of an electrolyte should influence the depressing effect; or, in other words, that the same valency rule should hold for the depressing effect of electrolytes on the cataphoretic P.D. as on the membrane potentials.

To test this possibility, the depressing effect of salts with monovalent and bivalent cations on the cataphoretic P.D. of particles of collodion, mastic, and graphite was compared. It was found that the valency rule holds. NaCl was used as a charging electrolyte for the particles of collodion, mastic, and graphite and it was used in that concentration in which its charging effect was about a maximum; namely, M/256. The solutions in these experiments had always a pH of 5.8; *i.e.*, they were as near the point of neutrality of water as was conveniently possible. At this pH the cataphoretic P.D. of collodion particles varied in M/256 NaCl between 52 and 47 millivolts and that of the mastic particles between 60 and 55 millivolts. In order to test the depressing effect of other chlorides, LiCl, KCl, RbCl, $MgCl_2$, $CaCl_2$, etc., 50 cc. of M/128 NaCl were mixed with 50 cc. of some other salt, *e.g.* M/8 LiCl. In this case the mixture consisted of M/256 NaCl and M/16 LiCl, where M/256 NaCl was considered the charging electrolyte and LiCl the depressing electrolyte. In all these experiments, NaCl was always present in the concentration of M/256 while the concentration of the depressing salts varied from M/16 to M/2,048. The cataphoretic P.D. was calculated with the aid of the Helmholtz-Perrin formula from measurements of the rate of migration of particles in an electrical field under the microscope in the way described in preceding papers.

Fig. 2 gives the depressing effect of ten salts, LiCl, NaCl, KCl, RbCl, $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, $MnCl_2$, and $CoCl_2$, on the particles of collodion charged to the maximum by M/256 NaCl. The abscissæ are the molar concentrations of these depressing salts and the ordinates are the cataphoretic P.D. in millivolts. The pH of all solutions was 5.8. It is obvious that the values for the depressing effect of the four salts with univalent cation, LiCl, NaCl, KCl, and RbCl, lie (within the limits of the accuracy of the experiments) on one curve; and that the values for the depressing effect of the chlorides with divalent cation lie also all on one curve, which is, however, consid-

erably lower than that for the monovalent cations. The results are so clear that they leave no doubt that the valency rule holds for the depressing effect of these salts on the cataphoretic P.D. of the negatively charged collodion particles.

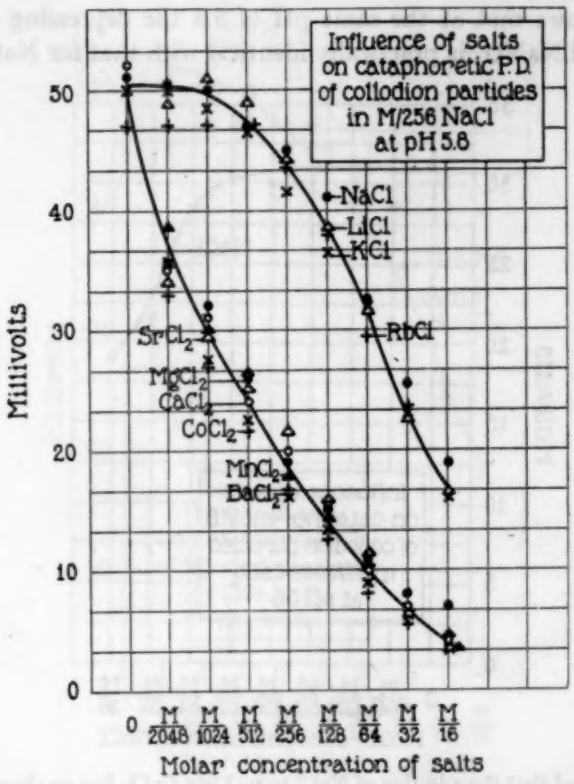

FIG. 2. Depressing effect of salts on cataphoretic P.D. of collodion particles in M/256 NaCl at pH 5.8. Only the valency but not the nature of the cation determines the depressing effect.

The maximal charging effect of $CaCl_2$ alone is reached at a concentration of about M/2,048, where the collodion particles have a P.D. of about 27 millivolts. When we add various concentrations of NaCl, the P.D. is never increased (Fig. 3) for the reason that the charging ion is the Cl ion and that the maximal charge possible by Cl ions in

the presence of Ca is already reached at a concentration of M/2,048 $CaCl_2$. This explains why the difference in the depressing effect between salts of the type of NaCl and CaCl is greater in the case of cataphoretic potentials than in the case of membrane potentials, as shown in a comparison between Figs. 1 and 2.

Fig. 4 shows that at the same pH of 5.8 the depressing effect of NaCNS and $NaNO_3$ is practically identical with that for NaCl.

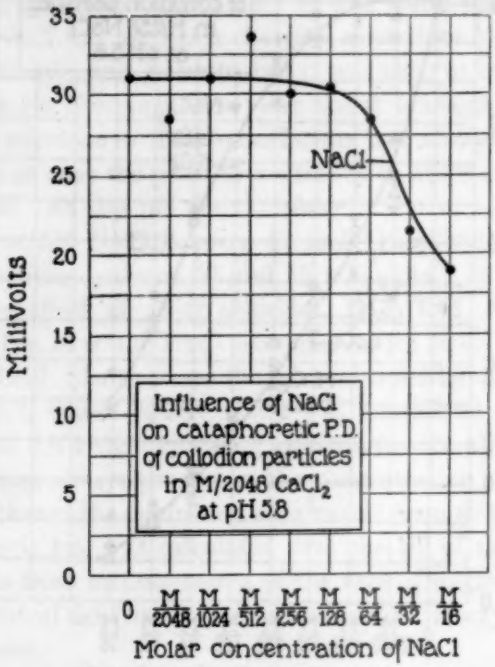

FIG. 3. Proof that the addition of NaCl to M/2,048 $CaCl_2$ has no charging effect on collodion particles.

It has been shown in preceding papers that there exists a critical P.D. below which suspensions are no longer stable and this P.D. is in the neighborhood of about 14 millivolts. This is true only for suspensions of hydrophobic colloids the stability of which is due exclusively to electrical double layers. The fact that the critical P.D. is almost the same, regardless of the nature of the particle or of the salt, leads to the assumption that the critical P.D. is that P.D. which no

longer suffices to prevent electrostatically the collision of particles moving with average velocity in the liquid. We should therefore expect that the precipitating concentration of all salts with univalent cations and univalent anions for collodion particles should be the same and that the same should be true for salts with divalent cations and uni-

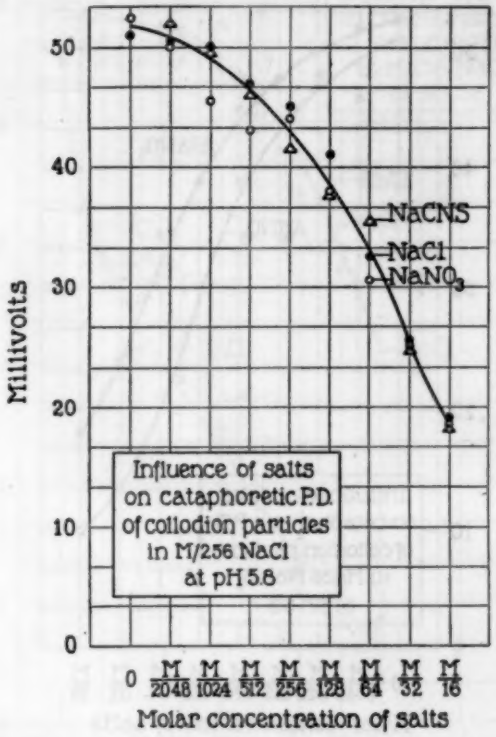

FIG. 4. Proof that the depressing effect of NaCl, $NaNO_3$, and NaCNS on the cataphoretic P.D. of collodion particles in M/256 NaCl is identical (pH 5.8).

valent anions, except that it should be lower for the latter than the former. In a preceding paper[7] it was shown that this postulate of the theory is fulfilled (for the same pH) for a number of hydrophobic colloids, *e.g.* collodion, mastic, gold, graphite, etc. There is only one exception. It has always been observed that the salts of heavy metals have a greater precipitating effect than salts of other cations of the

same valency; thus $AgNO_3$ has a greater precipitating effect than $NaNO_3$. Fig. 5 shows that $AgNO_3$ has a greater depressing effect on the cataphoretic P.D. of collodion particles than $NaNO_3$ at the same concentration and pH. This effect may or may not be due to the formation of complex salts by the ions of the heavy metals. In these

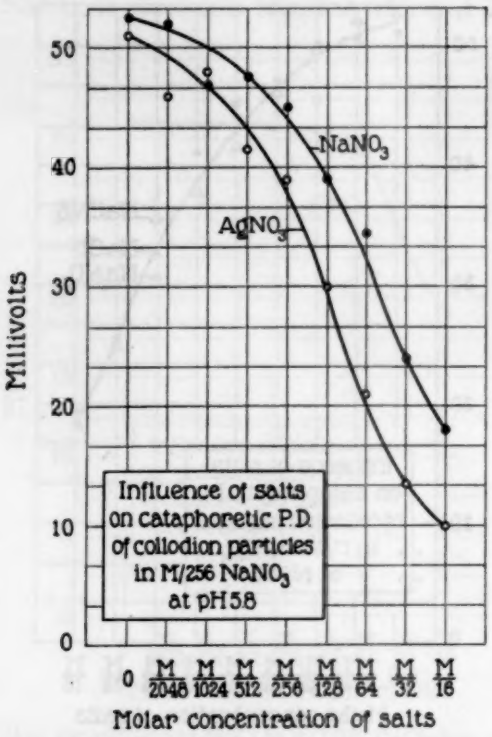

FIG. 5. Proof that the depressing effect of $AgNO_3$ on cataphoretic P.D. of collodion particles in M/256 $NaNO_3$ is greater than that of $NaNO_3$.

experiments M/256 $NaNO_3$ was used as charging salt instead of NaCl. Acids may also cause complications by reacting chemically with the particles.

Fig. 6 shows that the valency rule holds also for the depressing effect of salts on mastic particles as it does in the case of collodion particles. It may be added that similar results confirming the valency rule for

the depressing effect of salts on the cataphoretic P.D. were also obtained in the case of particles of Acheson's graphite.

These results, then, leave no doubt that the valency rule holds for the depressing effect of salts on the cataphoretic P.D. of non-electro-

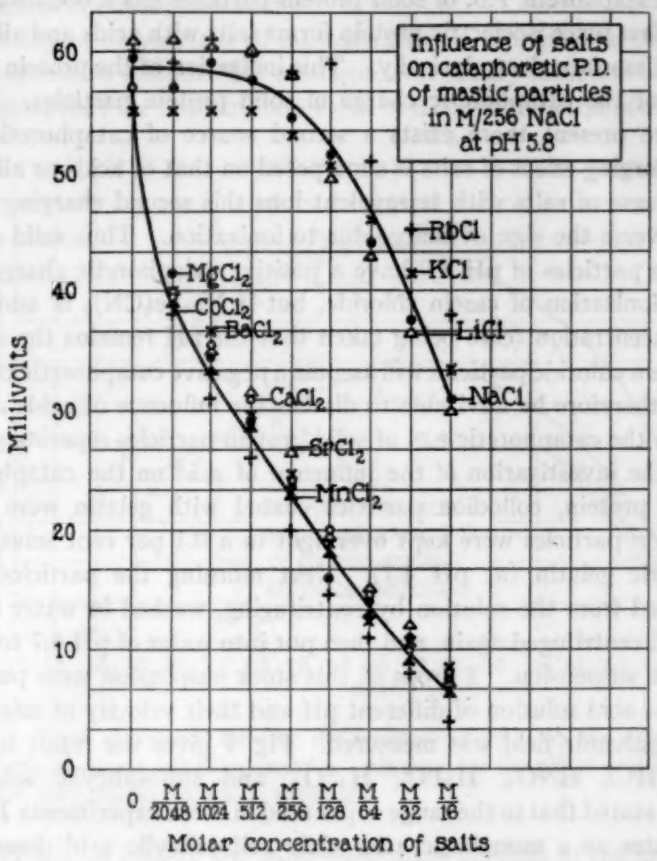

FIG. 6. Depressing effect of salts on the cataphoretic P.D. of mastic particles in M/256 NaCl at pH 5.8.

lytes; except in such cases where chemical complications may be expected. Unless it can be shown that the valency rule holds under other conditions than those of the Donnan equilibrium, these experiments confirm Wilson's hypothesis.

IV.

Proof of the Valency Rule for the Cataphoretic P.D. *of Solid Protein Particles.*

The cataphoretic P.D. of solid protein particles has a double origin. In the first place isoelectric protein forms salts with acids and alkalies, which dissociate electrolytically. This ionization of the protein is one source of the cataphoretic charge of solid protein particles. When salts are present there exists a second source of cataphoretic P.D. This charging effect of salts is superposed on that of acids or alkalies. In the case of salts with tetravalent ions this second charging effect may reverse the sign of charge due to ionization. Thus solid casein chloride particles of pH 4.0 have a positive cataphoretic charge, due to the ionization of casein chloride, but if $Na_4Fe(CN)_6$ is added in low concentration (care being taken that the pH remains the same), the casein chloride particles will assume a negative cataphoretic charge. It will therefore be advisable to discuss the influence of acids and of salts on the cataphoretic P.D. of solid protein particles separately.

For the investigation of the influence of acid on the cataphoretic P.D. of protein, collodion particles coated with gelatin were used. Collodion particles were kept overnight in a 0.1 per cent solution of isoelectric gelatin (at pH 4.7). Next morning the particles were separated from the solution by centrifuging, washed in water of pH 4.7, and centrifuged again, and then put into water of pH 4.7 to serve as stock suspension. 2 drops of this stock suspension were put into 50 cc. of acid solution of different pH and their velocity of migration in the galvanic field was measured. Fig. 7 gives the result for five acids, HCl, HNO_3, H_3PO_4, H_2SO_4, and sulfosalicylic acid. It may be stated that in the range of pH used in these experiments H_3PO_4 dissociates as a monobasic acid while sulfosalicylic acid dissociates like a strong dibasic acid. The abscissæ are the pH of the acid solutions in which the cataphoretic P.D. was measured, while the ordinates are the values of the cataphoretic P.D. in millivolts.

It is obvious that the five acids are separated into two distinct groups, one group including the three monobasic acids, HCl, HNO_3, and H_3PO_4, and the second group including the two strong dibasic acids, sulfuric and sulfosalicylic acid.

What is of importance is that the values for the three monobasic acids lie—within the limits of experimental accuracy—on one curve; and that the same is true for the two dibasic acids. This proves that only the valency but not the chemical nature of the anion of the acid determines the cataphoretic P.D.

The degree of accuracy of measurements is too small to permit us to lay stress on quantitative relations; and it may therefore be rather accidental that the approximate value of 0.66 for the ratio of the cataphoretic P.D. of dibasic and monobasic acids prevails at the same pH. This is the value demanded by the Donnan theory.[8,10] Omitting the values of P.D. below 10 millivolts, we find the following ratios (Table I).

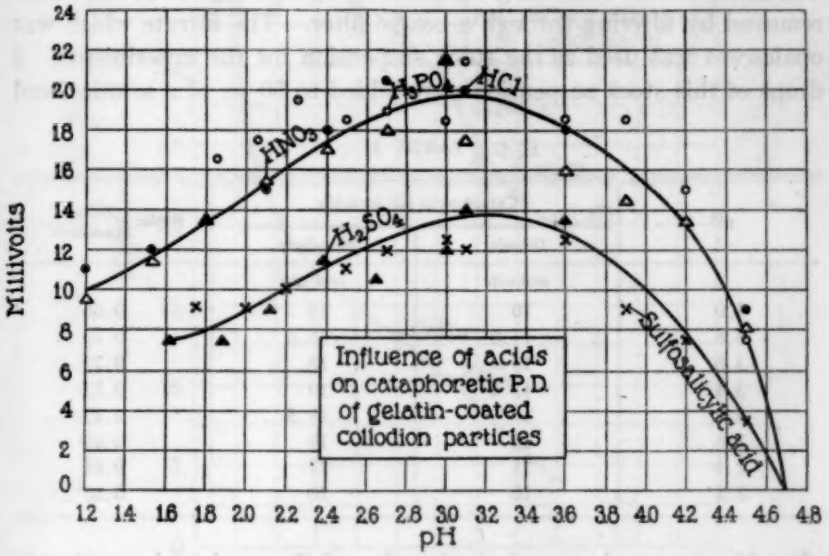

FIG. 7. Proof of valency rule for the influence of acids on the cataphoretic P.D. of gelatin-coated collodion particles. Abscisse are the pH of acid solutions, ordinates, the cataphoretic P.D. in millivolts.

Whatever may be the theoretical explanation of these measurements, it is certain that the forces due to the ionization of the protein

[10] Loeb, J., and Kunitz, M., *J. Gen. Physiol.*, 1922–23, v, 665.

itself outweigh in this case any influences which forces inherent in the water itself might have on the cataphoretic P.D. under the influence of acids.

The effect of salts on the cataphoretic P.D. of protein particles was tried on negatively charged particles of denatured egg albumin, to make the results comparable to the experiments on negatively charged particles of collodion and mastic. A 1 per cent solution of isoelectric crystalline egg albumin (pH = 4.8), prepared after Sörensen's method, was heated to 90°C. The gross particles of the coagulated mass were removed by filtering through a coarse filter. The filtrate which was opalescent was used as the stock suspension for the experiments. 3 drops of this stock suspension were added to 50 cc. of a solution and

TABLE I.

pH	Cataphoretic P.D. for acids.		Ratio: $\frac{\text{Dibasic}}{\text{Monobasic}}$
	Dibasic.	Monobasic.	
	millivolts	*millivolts*	
4.0	10	15	0.66
3.8	11.6	16.6	0.70
3.6	13	18	0.72
3.4	13.6	19	0.72
3.2	14	19.5	0.72
2.8	13	19	0.68
2.4	11	17	0.65
2.2	10	16	0.63

after 20 minutes the speed of migration of the particles in an electric field was measured.

We used as a charging electrolyte M/5,000 NaOH which transformed the isoelectric albumin into sodium albuminate (with negative albumin ions). The cataphoretic P.D. of the particles in this solution varied between 32 and 38 millivolts. In such solutions of M/5,000 NaOH were contained various salts, LiCl, NaCl, KCl, RbCl, $CaCl_2$, and $BaCl_2$, as depressing electrolytes in concentrations varying from M/2,048 to M/16. Fig. 8 gives the results, showing again a clear valency effect.

The salts with univalent cation, LiCl, NaCl, KCl, and RbCl, had an equal depressing effect on the cataphoretic P.D. of the particles

of denatured egg albumin, all the values lying on one curve. The values for the depressing effect of $CaCl_2$ and $BaCl_2$ also lie on one curve, which is, however, considerably lower than that for salts of the type NaCl. The curves in Fig. 8 resemble the corresponding curves in Fig. 2 for collodion particles and Fig. 6 for mastic particles.

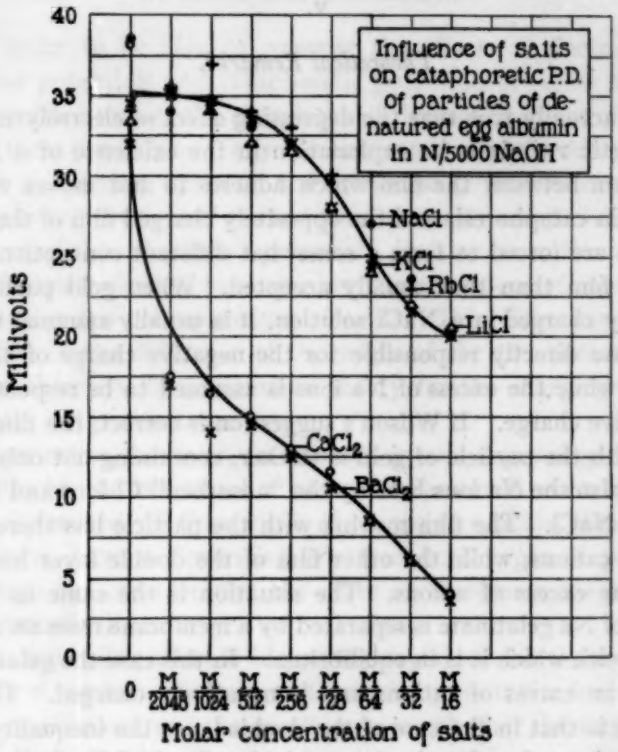

FIG. 8. Depressing effect of salts on the cataphoretic P.D. of denatured egg albumin.

We may therefore draw the conclusion that regardless of the chemical nature of the particles the depressing influence of electrolytes on the cataphoretic P.D. is determined by the valency but not by the nature of that ion of an electrolyte which has a sign of charge opposite to that of the particles. The only exceptions are where the electrolyte changes the chemical constitution of the particle (as possibly in the

case of heavy metals). In the case of the influence of acids on the cataphoretic P.D. of collodion particles coated with gelatin the cataphoretic P.D. seems to be determined by the ionization of the protein in a way as if the Donnan equilibrium might be the determining factor.

V.

Theoretical Remarks.

If it is actually true that the depressing effect of electrolytes on the cataphoretic P.D. finds its explanation in the existence of a Donnan equilibrium between the film which adheres to and moves with the particles in cataphoresis and the oppositely charged film of the double layer, we are forced to form a somewhat different conception of this adhering film than that usually accepted. When gold particles are negatively charged in a NaCl solution, it is usually assumed that the Cl ions are directly responsible for the negative charge of the gold particles while the excess of Na ions is assumed to be responsible for the positive charge. If Wilson's suggestion is correct, the film which moves with the particle of gold is thicker, containing not only the Cl ions but also the Na ions held by the "adsorbed" Cl ions and in addition free NaCl. The film moving with the particle has therefore an excess of cations, while the other film of the double layer has a corresponding excess of anions. The situation is the same as when a solution of Na gelatinate is separated by a membrane from an aqueous solution with which it is in equilibrium. In this case the gelatin solution has an excess of cations and is negatively charged. The only difference is that in the case of the double layer the inequality in the concentrations of cations in the enveloping film and the bulk of solution is not due to a membrane but to the forces which press the Cl ions in excess on the solid particle. This leads to an excess of cations in the film which adheres to and moves with the particle.

In his recent papers the writer has made use of the older conception of the double layer, but the proof of the validity of the valency rule for the depressing effect of ions on the cataphoretic P.D. forces us to consider Wilson's conception of the constitution of the electrical double layer.

The measurements of the rate of migration of the particles were carried out by the writer's assistant, Mr. M. Kunitz.

VI.

SUMMARY AND CONCLUSIONS.

1. In order to be able to compare the effects of electrolytes on membrane potentials and cataphoretic potentials it seems necessary to distinguish between the charging and depressing effect of electrolytes on these potentials. Only low concentrations of acids and alkalies have a charging effect on the *membrane* potentials of proteins, while low concentrations of neutral salts have only a depressing effect; in the case of the *cataphoretic* potentials, low concentrations of salts have a charging effect as have also low concentrations of alkalies and in some cases low concentrations of acids. This difference finds its explanation in the difference of the origin of the two potentials and there can therefore be no common theory for the charging effect of electrolytes in the two cases.

2. There exists, however, an analogy in the depressing action of electrolytes on the two types of potentials inasmuch when the maximal P.D. is reached, all three kinds of electrolytes, acids, alkalies, and neutral salts, have a depressing effect on both types of potentials (taking into due consideration the effect of changes in the hydrogen ion concentration).

3. This depressing effect is adequately explained for the membrane potentials of protein solutions and protein gels on the basis of the Donnan equilibrium, and the question arises whether the same explanation may also hold for the cataphoretic potentials.

4. The active ion in the depressing action of electrolytes on membrane potentials as well as on cataphoretic potentials has the opposite sign of charge from that of the colloidal particle. It had been shown before[5,10] that only the valency but not the chemical nature of the active ion determines the depressing effect in the case of membrane potentials and it is shown in this paper that the same is true for the cataphoretic potentials of particles of collodion, mastic, Acheson's graphite, and denatured egg albumin.

5. It is shown that the same valency rule holds also for the effect of acids on the cataphoretic potentials of collodion particles coated with gelatin, and that the ratio of the effect of dibasic to that of monobasic acids is approximately 0.66, as Donnan's theory of membrane potentials would demand.

6. If we have a right to conclude from the validity of the valency rule for cataphoretic potentials that the depressing effect of electrolytes on the cataphoretic P.D. is determined by the Donnan equilibrium, we can understand the analogy between the depressing action of electrolytes on membrane potentials of hydrophilic colloids and on the cataphoretic potentials of hydrophobic colloids. We can also understand the analogy between the influence of electrolytes on the precipitation of hydrophobic colloids and on the depression of the values of all those properties of hydrophilic colloids which depend on the Donnan equilibrium, since the precipitation of hydrophobic colloids occurs when the cataphoretic P.D. is depressed below a critical value.

THE RATE OF GROWTH OF THE DAIRY COW.

V. Extrauterine Growth in Linear Dimensions.

By SAMUEL BRODY and ARTHUR C. RAGSDALE.

(From the Department of Dairy Husbandry, University of Missouri, Columbia.)

(Received for publication, November 24, 1923.)

The curves of growth of animals which have been studied—including that of the dairy cow[1]—show a number of more or less distinct growth cycles or growth epochs. The mathematical analyses of these cycles or epochs have proved very productive.[2] On the other hand, relatively little has been accomplished in the mathematical analysis of the growth curve as a whole. The object of this communication is to briefly present the results of a study of the extrauterine growth of the dairy cow in linear dimensions with the object of formulating an equation or a law representing the *general* course of growth as contrasted to the equations representing growth cycles or growth epochs.

The data on which this study is based have been accumulating in this department for some time in connection with an investigation concerning the effect of the age of first calving, and the liberality of the food supply on the growth of the dairy cow. The methods of measurements and other details of this investigation, which were formulated and which were carried on for a considerable period of time by Eckles and his students, are discussed in detail in a bulletin of this station.[3] Mr. Swett of this department has also made a

[1] Brody, S., and Ragsdale, A. C., *J. Gen Physiol.*, 1920–21, iii, 623.

[2] Robertson, T. B., The chemical basis of growth and senescence, Monograph on experimental biology and medicine, Philadelphia and London, 1923. Ostwald, W., Vorträge und Aufsätze über Entwicklungsmechanik der Organismen, Leipsic, 1908, No. 5. Raudnitz, R. W., *Prag. med. Woch.*, 1892, xvii, 67, 82. Wiener, C., Über das Wachstum des Menschlichen Körpers, Karlsruhe, 1890. von Lange, E., *Jahrb. Kinderh.*, 1903, lvii, 261.

[3] Eckles, C. H., and Swett, W. W., *Univ. Missouri Agric. Exp. Station Research Bull. 31*, 1918. See also Eckles, C. H., *Univ. Missouri Agric. Exp. Station Research Bull. 36*, 1920.

considerable contribution to these data in connection with an investigation which is being carried on concerning the minimum protein requirements for cattle during growth. For present purposes the details of this investigation may be summarized by stating that twenty-one linear measurements were taken of the cows at monthly intervals during the first 60 months of extrauterine life. The animals under observation consisted of Jersey, Holstein, Ayrshire, and a few milking Shorthorn cattle. Each breed was divided into subgroups according to the age of first calving, and the liberality of the ration they received.

All these data have been prepared for publication in connection with this study. However, the magnitude of these data makes their publication impractical. In this communication, therefore, the results for only one breed, that of Jersey cattle (average of all subgroups), are presented, and these only graphically as shown in Fig. 1. To avoid confusion only eleven of the twenty-one measurements are plotted in this figure.[4]

In Fig. 1 are given the observed values and also curves representing the equation

$$Y = A - Be^{-k(t+9.4)} \qquad (1)$$

which was fitted to the data. In this equation Y represents the values of the measurements at the ages, t (months). The value 9.4 in the exponent represents the time in months of intrauterine life of this animal, e, the base of natural logarithms, A, a constant representing the values of the measurements at maturity, and B is another constant. The minus sign in the exponent indicates, of course, that the speed of growth decreases with age. The agreement between observed and computed values is seen to be fairly satisfactory.

The significance of this fairly satisfactory agreement between observed and computed values, consists principally in the fact that this agreement can probably be interpreted on a rational basis. Equation (1) from which the curves were plotted, has the same form

[4] Copies of all the numerical data of this investigation will be deposited in the University of Missouri Library for the benefit of those who may desire to make use of this material for other studies on growth.

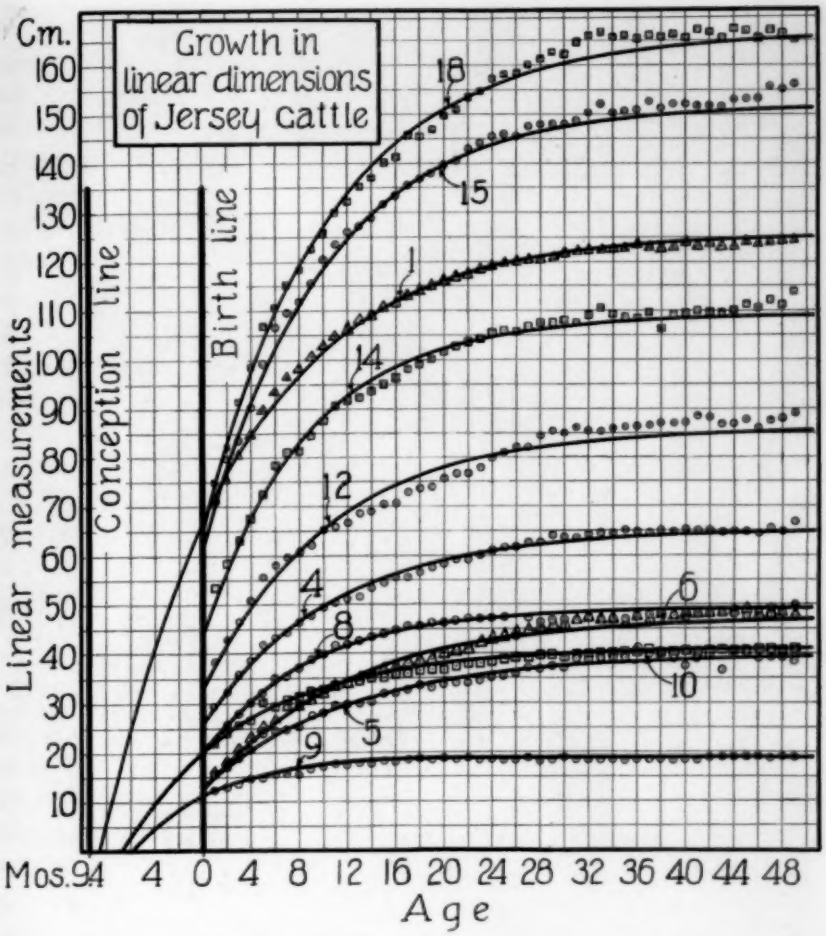

FIG. 1. Growth in linear dimensions of Jersey cattle. The smooth curves passing through the observed values represent the equation

$$Y = A - Be^{-k(t+9.4)}.$$

The numerals refer to the measurements as indicated in Fig. 2, as follows: 18 heart girth, 15 from point of shoulder to ischium, 1 height at withers, 14 from point of shoulder to a point of hips, 12 from highest point of withers to a line between hips, 4 depth of chest just behind elbow joint, 8 length from poll to point of muzzle, 6 width of hips, 10 circumference of muzzle at opening of mouth, 5 width of chest just behind elbow joint, and 9 width of forehead.

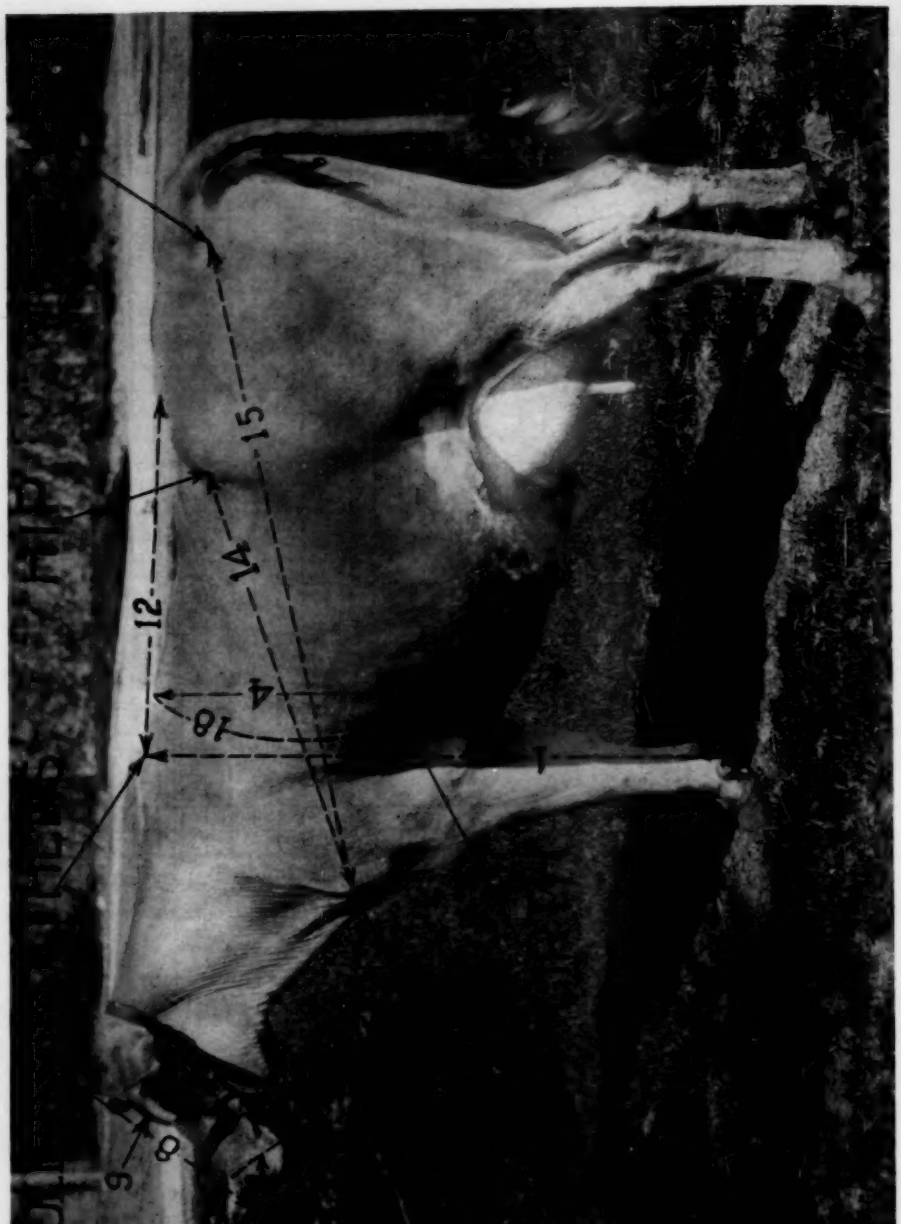

Fig. 2. The points of anatomy, and the measurements taken of the cows referred to in Fig. 1.

as the equation of a monomolecular chemical reaction. This naturally suggests the interpretation that the fundamental course of growth is limited by a monomolecular process, and that the cyclic phenomena are subsidiary processes in the fundamentally exponential course of growth.

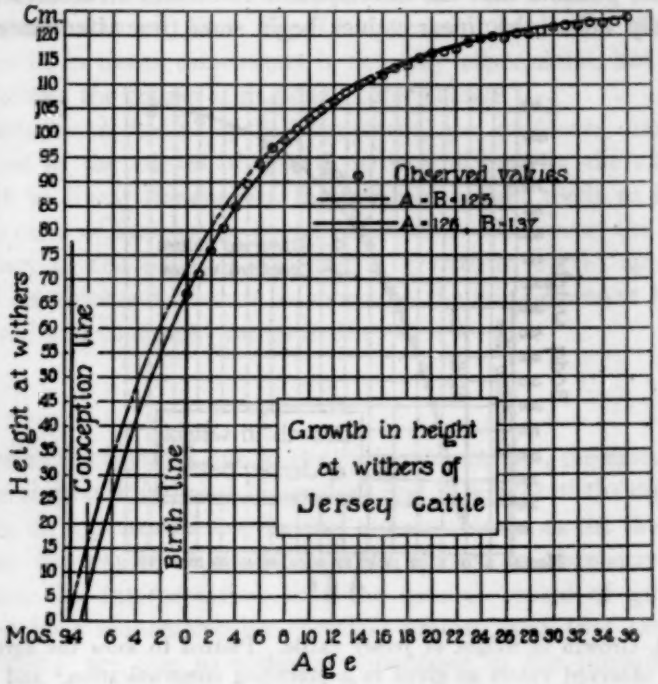

FIG. 3. Growth in height at withers plotted to show the relative agreement between observed and computed values using the formulæ

$$Y = 125.9 - 137.4e^{-0.09(t+9.4)}$$

and

$$Y = 125 - 125e^{-0.09(t+9.4)}$$

that is when A and B have different values, and when they have the same values.

Equation (1) differs from the equation of a monomolecular reaction only in the fact that in this equation the numerical values of the constants A and B differ, while in the equation of a monomolecular

FIG. 2. The points of anatomy, and the measurements taken of the cows referred to in Fig. 1.

change they are the same. When A and B have the same value the curves begin at zero; if A and B differ, the theoretical curve does not begin at zero. As seen in Fig. 1, the computed values do not begin at conception, which is presumably the beginning of growth in linear dimensions, but some time between conception and birth. It is, of course, possible that the development of certain structures which materially affect the linear values begin some time after conception

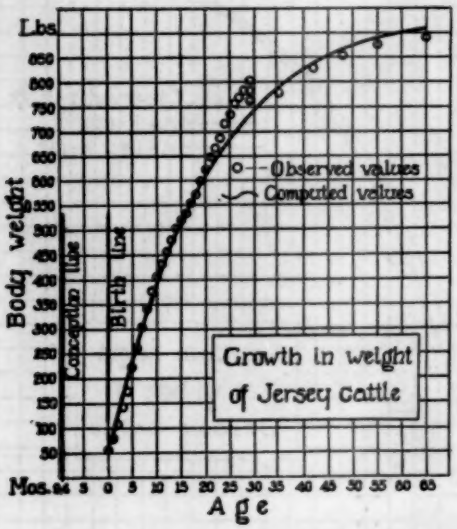

FIG. 4. Growth in weight of Jersey cattle. Plotted to show the agreement between observed values as given in a preceding communication,[1] and values computed from the exponential equation

$$Y = 950 - 925e^{-0.054}.$$

The rise between 20 and 29 months is due to gestation, and the sudden decline at about 29 months is due to calving. The animals were bred at about 20 months of age and calved at about 29 months of age.

as shown by the curves. This possibility cannot, of course, be present as regards growth in weight (Fig. 4). It may also be that the beginning of the growth process is irregular in the sense that the course of the process at the beginning of growth is somewhat different from that of the course of the process after it is well started.

Such irregularities at the beginning of a process are of frequent occurrence in chemistry. The relation between the curves of height at withers as plotted from equation (1) in which the numerical values of A and B differ, and the same equation in which the numerical values of A and B are the same, are shown in Fig. 3. From Fig. 1 it is clear that the extrauterine period of growth certainly follows an exponential course. From Fig. 3 it appears probable that the course of growth in linear dimensions is not only exponential, but that it also follows the course of monomolecular change.

Fluctuations due to cyclic phenomena are, of course, more pronounced in the curves of growth in weight than in the curves of growth in linear dimensions, inasmuch as weight tends to increase as the cube of increase in linear dimensions. However, the general tendency of the curve of extrauterine growth in weight appears to follow an exponential course as shown by the fair agreement between observed and computed values in Fig. 4.

SUMMARY.

Barring fluctuations due to the cyclic phenomena, the extrauterine course of growth in linear dimensions and in weight of the dairy cow follows an exponential law having the same form as the law representing the course of monomolecular change in chemistry. This suggests the interpretation that the general course of growth is limited by a monomolecular chemical process, and that the cyclic phenomena are due to subsidiary processes in the fundamentally exponential course of growth.

The fact that growth follows or tends to follow an exponential course may be stated more simply as follows: if the unit of time is taken sufficiently large so that fluctuations due to the cyclic phenomena are balanced or eliminated, then the amount of growth made during the given unit of time at *any* age tends to be a constant percentage of the growth made during the preceding unit of time. Thus, the growth in height at withers made during any year is about 34 per cent of the growth made during the preceding year. Similarly the growth in weight made during any year is about 56 per cent of the growth in weight made during the preceding year. This is in

accordance with expectations if it is assumed that each animal begins life with a definite endowment of limiting substance necessary for the process of growth, and that this endowment is used up at a constant rate (or percentage) of itself.

A TEST FOR DIFFUSIBLE IONS.

I. The Ionic Nature of Trypsin.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication November 20, 1923.)

The ionization of a substance which may be prepared in any reasonable degree of purity may be easily shown by the conductivity method. In the case of substances, however, which cannot be isolated, the proof of the existence of ions is difficult. The method of migration in an electric field is uncertain, owing to the fact that particles of practically any material show movement under these conditions and that such movement does not necessarily indicate the existence of ions.

The theory of membrane equilibrium advanced by Donnan,[1] however, predicts conditions which offer a criterion for the ionic nature of a substance. Donnan showed by thermodynamic reasoning that, if a solution containing a mixture of diffusible and non-diffusible ions was separated by a membrane from another solution containing only diffusible ions, the concentration of diffusible ions would be different on the two sides of the membrane. At equilibrium the product of the concentration for any one pair of oppositely charged diffusible ions of the same valence on one side of the membrane must be equal to the product of the concentrations of the same pair on the opposite side of the membrane. It follows that the ratio of the concentration of any pair of negative ions inside to that outside must be equal to the ratio of the concentrations of any pair of positive ions of the same valence outside to that inside the membrane.

[1] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

Expressed mathematically, the equation is

$$(A_o^{-n})^{1/n}(B_o^{+m})^{1/m} = (A_i^{-n})^{1/n}(B_i^{+m})^{1/m}$$

or

$$\frac{(A_o^{-n})^{1/n}}{(A_i^{-n})^{1/n}} = \frac{(B_i^{+m})^{1/m}}{(B_o^{+m})^{1/m}} = \frac{(C_i^{+l})^{1/l}}{(C_o^{+l})^{1/l}} = \cdots\cdots$$

in which (A_i) is the concentration of an n valent negative ion inside the membrane, (A_o) is the concentration of the same ion outside. B, C, etc., are any other diffusible ions present, having the valence m, l, etc. It can further be shown that if the non-diffusible ion is positive, the concentration of diffusible positive ions will be greater outside the membrane, and that, as the total concentration of diffusible ions increases, the difference in concentration inside and outside decreases and approaches zero. The same equation had apparently been derived, also thermodynamically, by Gibbs.[2] It has been experimentally verified by Procter and Wilson,[3] and very thoroughly by Loeb,[4] so that the equation rests on a substantial basis both theoretically and experimentally. In order to test the ionic nature of a substance therefore it is only necessary to set up such an equilibrium system, measure the concentrations of some ion such as hydrogen or chloride, and compare this ratio with the concentration ratio of the substance under investigation. The only difficulty lies in the fact that the equation predicts only the concentration of the ions and not the total concentration, so that if the substance is not completely ionized or is combined in non-ionic form in the solution, the determination of the total concentration will not lead to the correct ratio. In other words, if the experimental results do not agree with the ratio, the discrepancy may be due to complicating factors and no definite conclusion can be drawn, whereas if they do agree, the conclusion seems justified that the substance is ionic.

[2] *Cf.* Adair, G. S., *Science*, 1923, lviii, 13.

[3] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 309.

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

The above method has been applied to the distribution of trypsin and it has been found that trypsin behaves like a monovalent positive ion from pH 2 to 10.2. At this point it behaves as though it were unionized and on the alkaline side of 10.2 becomes a monovalent negative ion. The experiments in this strongly alkaline range, however, are not so satisfactory.

Experimental Procedure.

Owing to the unstable nature of trypsin, it is necessary to provide conditions under which equilibrium may be rapidly established. This can be done by using finely powdered gelatin. The fact that trypsin attacks gelatin might be expected to cause a complication. This was avoided as much as possible by working at 0°, and no evidence was found to show that the action of the trypsin on the gelatin had any effect on the experiment. 2 gm. of dry isoelectric gelatin, passing an 80 sieve, were placed in 45 cc. of solution and kept at 0° about 18 hours. This is more than sufficient for equilibrium to be reached. 1.0 cc. of a dilute trypsin solution was then added, the total volume made up to 50 cc., and the suspension allowed to stand 2 hours longer at 0°. The gelatin was then quickly filtered off and the volume of filtrate measured. In preliminary experiments the hydrogen ion concentration of the solution and of the gelatin (after melting) were measured. This procedure is difficult, however, and it was found much more accurate and convenient to measure the chloride concentration of the filtrate. The concentration of the chloride ion in the gelatin was then found by difference. The ratios for the Cl concentration found in this way agreed with the H ratios as Loeb has found and in the experiments reported here the Cl ion has been taken as the indicator of the equilibrium. The Cl was determined by $AgNO_3$ titration. The trypsin concentration of the filtrate and gelatin was determined by the viscosity method described by Hussey and the writer.[5] In order to make this determination, it was necessary to melt the gelatin and under some conditions there is danger of destruction of the trypsin by this process. A control experiment without gelatin was therefore made and the total amount of trypsin determined

[5] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

from this solution. If no disturbing factor had occurred, therefore, the total amount of trypsin found in the experiment should agree with the amount found in the control.

Preparation of Trypsin.—A 2 per cent solution of Fairchild's trypsin was prepared and brought to pH 4.5 with HCl. A heavy precipitate of protein forms under these conditions. This was filtered off and the solution brought back to pH 7.4. It was kept at 0° for 2 or 3 days. At the end of this time the solution gave no precipitate with trichloroacetic acid and was therefore presumably free from protein. Some experiments were made with trypsin which had been dialyzed through collodion. There was no difference in the behavior of these solutions.

TABLE I.

Time for Equilibrium.

2 gm. of gelatin + 200 cc. of M/100 phosphate buffer, pH 7.0. Kept at 0° for 18 hours and 0.2 cc. of 5 per cent trypsin added. Samples were removed after the time interval noted, filtered, and trypsin determined in filtrate.

Time interval	Trypsin units / 0.2 cc. filtrate
hrs.	
0	3.7
0.02	1.2
0.30	0.47
1.10	0.45
	0.48

Time Required for Equilibrium.—Since Donnan's equation only holds at equilibrium, it is necessary to be sure that sufficient time is allowed before analyzing the solution. Table I shows that equilibrium is reached in less than 1 hour. The solutions were allowed to stand 2 hours, however, and on the acid side, where the trypsin is more stable and no action on the gelatin occurs, they were kept at 0° for 18 hours after the addition of the enzyme.

Experimental Results.

The details of a single experiment are given in Table II. The ratio of the trypsin concentration inside to that outside the gelatin is

evidently nearly equal to the chloride ratio outside to that inside. The trypsin must, therefore, be of the opposite sign from that of chloride and be monovalent. This experiment was then repeated at a series of different pH. On the acid side the pH was regulated simply by the addition of HCl, while on the alkaline side the chloride ion was furnished by the addition of M/100 KCl and the pH regulated by NaOH. From pH 6 to 11 there was present in addition M/100 sodium borate to act as a buffer. This depresses the equilibrium and accounts for the fact that the ratio does not increase beyond pH 6.

The results of these experiments are shown in Table III. Each figure is the average of two to ten experiments. The table shows

TABLE II.

Data for Trypsin and Chlorine Distribution.

2 gm. of gelatin added to 45 cc. of N/50 HCl at 0°C. Kept at 0° for 18 hours. 1 cc. of 2 per cent trypsin added, volume made up to 50 cc., and solution stirred occasionally. After 2 hours gelatin filtered off, and the volume of the filtrate, Cl concentration, and trypsin concentration determined.

Total volume, *cc.*	50	Units trypsin/cc. of filtrate	8.3
Total Cl, *cc.* of M/30	29	Units trypsin/cc. of gelatin	1.3
Total filtrate, *cc.*	17	Total trypsin { Control	189
Cl/cc. filtrate, *cc.* of M/30	0.14	Total trypsin { Experiment	184
Total Cl filtrate, *cc.* of M/30	2.40	Ratio: $\frac{\text{Trypsin gelatin}}{\text{Trypsin liquid}}$	0.156
Total Cl gelatin, *cc.* of M/30	26.6		
Volume gelatin, *cc.*	33	Ratio: $\frac{\text{Cl liquid}}{\text{Cl gelatin}}$	0.17
Cl/cc. gelatin, *cc.* of M/30	0.81		

that the trypsin ratio agrees with the chloride ratio throughout except near pH 4.7 and near 10.2. They further show the characteristic maximum at about pH 3.5 and the change in the value of the ratio from more than 1 on the alkaline side of the isoelectric point of the gelatin, to less than 1 on the acid side. From pH 4.5 to about 6.0 all the trypsin solutions used showed a slight precipitate, especially in the presence of gelatin. This precipitate contains trypsin and is probably the cause of the irregular results contained in this range of pH. The amount of trypsin in this precipitate becomes included in the trypsin found in the gelatin and makes the figure too high. The same effect was occasionally noted with very high salt concentrations.

When high concentrations of salt were used, so that the Cl^- ion distribution was equal on both sides, it was occasionally found that the trypsin was more concentrated in the gelatin. In these experiments, as in those near the isoelectric point, the volume of gelatin is small (10 to 12 cc.). It is possible that a small amount of trypsin is combined with the gelatin and that this is the disturbing factor.

TABLE III.

Effect of pH on Distribution of Chloride Ions and Trypsin, Inside and Outside of Gelatin Particles.

pH	2.0	2.5	3.0	3.5	4.0	4.7	5.5	6.0	6.5
Ratio: $\frac{Cl^-\text{ conc. liquid}}{Cl^-\text{ conc. gelatin}}$	0.60	0.40	0.24	0.13	0.38	1.0	1.3	1.6	2.0
Ratio: $\frac{\text{Trypsin conc. gelatin}}{\text{Trypsin conc. liquid}}$	0.50	0.30	0.23	0.17	0.35	1.5 11.0 1.6 20.0	1.5 4.0 6.0 1.2	1.4	2.5
pH	7.0	8.0	8.5	9.0	9.2	9.4	9.6	9.8	10.0
Ratio: $\frac{Cl^-\text{ conc. liquid}}{Cl^-\text{ conc. gelatin}}$	1.9	1.9	1.8	2.0	2.4	2.6	2.5	2.2	2.3
Ratio: $\frac{\text{Trypsin conc. gelatin}}{\text{Trypsin conc. liquid}}$	1.9	1.7	2.1	2.0	2.6	2.5	2.6	2.0	1.9
pH	10.2	10.4	10.6	10.8	11.0				
Ratio: $\frac{Cl^-\text{ gelatin}}{Cl^-\text{ liquid}}$	0.45	0.55	0.50	0.50	0.45				
Ratio: $\frac{\text{Trypsin gelatin}}{\text{Trypsin liquid}}$	1.0	0.38	0.43	0.43	0.30				

When the volume of gelatin is large, this factor would be negligible, but under such conditions as to destroy the Donnan equilibrium and prevent the swelling of the gelatin a small amount of combined trypsin would cause a larger error. This discrepancy near the isoelectric point of gelatin may be largely avoided by allowing the gelatin to stand for 48 hours at pH 3.5 and then adjusting the pH to 4.7.

The experiments as a whole in this range indicate that gelatin may combine with trypsin and that this effect is much greater when the gelatin is not completely permeated with water.

The table shows further that on the acid side of the isoelectric point the trypsin is more concentrated in the liquid while on the alkaline side it is more concentrated in the gelatin. This effect, however, would be common to any positive ion under the conditions of the experiment, and is not due to any peculiar relationship of the gelatin and trypsin. At pH 10.2 the trypsin is equally distributed in the gelatin and liquid and on the alkaline side of this point becomes distributed in the same way as the chloride instead of the reciprocal. This would be the result expected if the trypsin were amphoteric with an isoelectric point at 10.2 and became a negative ion on the alkaline side of this point. There is no evidence of an isoelectric point at or near pH 4 as found by Michaelis and Davidsohn.[6] It seems possible that the migration of trypsin described by these writers was due to the carrying of the enzyme by some impurity in the solution as was apparently the case with pepsin.

Another characteristic of the Donnan equilibrium is the depressing effect of salts. The result of experiments in which the total salt concentration is varied is given in Table IV. The chloride and trypsin ratios are again in quite close agreement. It will be noted that on the alkaline side the concentration of the trypsin in the gelatin is decreased by the salt whereas on the acid side it is increased. This is typical of the Donnan equilibrium and is difficult to account for even qualitatively on any other basis. The volume of gelatin is decreased in both cases by the salt, so that if the trypsin were actually combined with the gelatin by any chemical mechanism or even by adsorption, the total amount combined would be constant (since the weight of gelatin is the same), and the concentration would therefore increase whenever the volume of gelatin decreased. This is not the case, however. On the alkaline side, decreasing the volume of gelatin also decreases the concentration of trypsin in the gelatin.

These and similar experiments were repeated a number of times with results that agreed more or less closely with the theory. It is impos-

[6] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxvi, 280.

sible to secure accurate results in a single experiment of this type, owing to the large experimental errors. All the experiments may be

TABLE IV.

Effect of Increasing Salt Concentration.

pH 8.5

2 gm. of gelatin, 7 cc. of 0.1 N NaOH, 2 cc. of 0.1 N KCl + increasing concentrations of Na_2SO_4. Total volume 50 cc.

Total salt concentration.	Ratio: $\frac{\text{Cl liquid}}{\text{Cl gelatin}}$.	Ratio: $\frac{\text{Trypsin gelatin}}{\text{Trypsin liquid}}$.
M		
0.024	4.2	3.6
0.06	2.3	2.4
0.10	2.2	2.0
0.18	2.1	2.0
0.34	1.3	1.8

pH 3.5

2 gm. of gelatin, 10 cc. of 0.1 N HCl, and increasing amounts of KCl.

Total Cl concentration.	Ratio: $\frac{\text{Cl liquid}}{\text{Cl gelatin}}$.	Ratio: $\frac{\text{Trypsin gelatin}}{\text{Trypsin liquid}}$.
0.02	0.15	0.14
0.04	0.35	0.28
0.06	0.43	0.35
0.08	0.49	0.40

pH 3.5

Increasing $NaNO_3$.

Conc. $NaNO_3$.		
0	0.14	0.13
0.02	0.25	0.30
0.04	0.30	0.34
0.08	0.41	0.39
0.16	0.50	0.65

used, however, by comparing the trypsin ratio for any experiment with the corresponding chloride ratio. According to the theory these ratios should be equal, that is, the chloride ratio divided by the tryp-

sin ratio should equal 1.00. The result of averaging the results in this way are shown below.

$$\frac{\text{Chloride concentration in liquid}}{\text{Chloride concentration in gelatin}} = \frac{\text{Trypsin concentration in gelatin}}{\text{Trypsin concentration in liquid}}$$

Calculated.....1.00
Observed (average of 106 experiments)...1.04 ± 0.03 (probable error of the mean).

The calculated ratio agrees with the experiment well within twice the probable error so that the result is statistically reliable. All the experiments were included in this average except those near pH 5.0 and 10.2, where there is evidently some complicating factor.

The experiments described above show clearly that the distribution of trypsin inside and outside of gelatin particles is regulated by the same forces that control the distribution of the hydrogen and chloride ions; in other words, by the Donnan equilibrium. Trypsin is therefore a diffusible ion and since the ratios are the reciprocal of the chloride ratios, it is a positive monovalent ion from pH 2 to 10.2, at which point it becomes isoelectric to become a negative ion in more strongly alkaline solution. It follows also that trypsin must be a fairly strong acid and base since the existence of any appreciable amount of unionized trypsin would disturb the ratios, owing to the equal distribution of the unionized portion. Since the method used determines the total amount of trypsin present, the presence of unionized trypsin would cause all the ratios to be nearer 1.0 than the chloride ratios; a result which is not obtained except at and near pH 10.2. This is presumably the isoelectric point.

These results further show that the removal of trypsin from solution by insoluble proteins is not evidence of the existence of a compound between the enzyme and the protein, as the writer formerly supposed,[7] but on the contrary proves that there is no combination between the two.

The numerous experiments showing a relation between the swelling of insoluble proteins and their rate of digestion, which have frequently

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

been cited[8] as evidence for some relation between the rate of hydrolysis and the hydration of the protein are also explained. They are simply due to the fact that the greater the inequality of the ion concentration the greater the swelling and also the greater the concentration of the trypsin inside the protein particle. The more a protein swells, therefore, on the alkaline side of its isoelectric point, the greater will be the concentration of the enzyme inside the protein and the more rapidly it will digest. The mechanism evidently has nothing to do with the hydration of the protein and in any case is secondary to the chemical effect of the pH on the protein. In the case of pepsin, for instance, the optimum for the digestion of gelatin by pepsin is not at pH 3.5, where the maximum swelling occurs, but at 2.5. This conclusion is also borne out by the fact that the pH effect is presented when a solution of the protein is used. With insoluble proteins, however, the purely chemical effect of the pH may be modified by the distribution effect.

The fact that trypsin passes through a collodion membrane had been noted by Strada[9] and also by Neill.[10] The experiments have been repeated and confirmed by the writer.

The concentration of trypsin in gelatin on the alkaline side of pH 4.7 and in the liquid on the acid side of this point offers a method for the purification of the enzyme. If trypsin is added to a suspension of gelatin particles at pH 7.0, most of the trypsin will be concentrated inside the gelatin. The particles may then be washed and then brought to pH 3.5, where the trypsin will diffuse out and become more concentrated in the liquid. Any diffusible positive ions would be carried along, but proteins should be largely removed.

It had been found by the writer that trypsin enters into equilibrium with the products of hydrolysis and that these equilibria were rapidly and completely reversible. Such equilibria are much more common between ions than between unionized substances, so that the behavior of trypsin in this case also indicates its ionic nature. Most cases of homogeneous catalysis are also due to ions so that it would not be surprising if such powerful catalysts as the enzymes were ionic.

[8] Ostwald, W., *Kolloid-Z.*, 1922, xxx, 234.
[9] Strada, F., *Ann. Inst. Pasteur*, 1908, xxii, 982.
[10] Neill, A. J., *Am. J. Physiol.*, 1921, lvii, 478.

Preliminary experiments indicated that pepsin is a monovalent negative ion.

SUMMARY.

1. The Donnan equilibrium furnishes a test for the ionic nature of any diffusible substance, since the ratio of the concentration of any ion on the two sides of a membrane must be equal to the ratio of the concentrations of any other ion of the same sign and valence, whereas a non-ionic substance would be equally distributed on both sides.

2. The distribution of trypsin inside and outside of gelatin particles has been compared to the distribution of hydrogen and chloride ions under the same conditions.

3. The ratio of the trypsin concentration in the gelatin to the concentration in the outside liquid is equal to the ratio of the hydrogen ion under the same conditions and to the reciprocal of the chloride ion ratio.

4. This result was obtained between pH 2.0 and 10.2. At pH 10.2 the trypsin is equally distributed and on the akaline side of 10.2 the ratio is directly equal to the chloride ratio.

5. Trypsin is therefore a positive monovalent ion in solutions of pH 10 to 2. It is probably isoelectric at 10.2 and a monovalent negative ion on the alkaline side of 10.2

6. Trypsin must also be a strong base since there is no evidence of any undissociated form on the acid side of pH 10.2.